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ROCHALI-
MAEA
HENSELAE

ROCHALI-
MAEA
VINSONII

ROCHALI-
MAEA
QUINTANA

RICKETT-
SIA PRO-
WAZEKII

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(57) Abstract

A method of diagnosing cat scratch disease and a method of diagnosing bacillary angiomatosis in a subject by detecting the presence of *Rochalimaea henselae* or an antigenic fragment thereof in the subject is provided. Also provided is a vaccine comprising an immunogenic amount of a nonpathogenic *Rochalimaea henselae* or an immunogenically specific determinant thereof and a pharmaceutically acceptable carrier. A method of diagnosing *Rochalimaea quintana* infection in a subject by detecting the presence of a nucleic acid specific to *Rochalimaea quintana* in a sample from the subject is provided. A purified, 60-kDa heat shock protein of *Rochalimaea* is provided. Also provided is a 17-kDa antigenic polypeptide of *Rochalimaea*.

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NUCLEIC ACIDS OF *ROCHALIMAEA HENSELAE* AND *ROCHALIMAEA*
QUINTANA AND METHODS AND COMPOSITIONS FOR DIAGNOSING
ROCHALIMAEA HENSELAE AND *ROCHALIMAEA QUINTANA* INFECTION

5

BACKGROUND OF THE INVENTION

Cat scratch disease (CSD) has been the subject of considerable clinical and microbiologic interest for many years. An estimated 7,000 cases of cat scratch disease occur each year in the United States. Due to difficulty in diagnosing CSD and its potentially confusing clinical similarity with other disease syndromes, the number of actual cases of CSD in the United States may be closer to 70,000 per year. CSD is described as a subacute regional lymphadenitis temporally associated with the scratch or bite of a cat, and it occasionally results in meningoencephalitis.

Diagnosis of CSD has been a problem because the etiologic agent of the disease has not been previously identified. An unidentified bacillus has been visualized in biopsies from patients with CSD using Warthin-Starry stain but has resisted identification because of difficulties in obtaining an isolated culture. The etiologic agent of CSD has recently been proposed to be "*Afipia felis*" (7). Despite these efforts, it has not been possible thus far to isolate or otherwise associate this agent with most persons suffering from cat scratch disease.

30

A clinically related disease, bacillary angiomatosis (BA), is a condition characterized by multiple tumors or swelling due to proliferation of the blood vessels. BA is often found in association with an immunocompromised condition, particularly HIV infection. An unidentified bacillus has been visualized in the angiomatous tissues using Warthin-Starry stain (28). DNA extracted from the

angiomatous tissues was shown to contain a fragment of 16S rRNA gene related to, but not identical to, the 16S rRNA gene of *Rochalimaea quintana*. This DNA was not obtained from a pure culture of the organism (28). These investigators were unable to isolate an infectious organism from patient tissues and, therefore, were unable to clearly associate the DNA sequences observed in tissues with an identifiable disease-causing organism. Neither the organism seen in these tissues nor the actual causative agent of the disease was identifiable.

Thus, despite intensive research and widespread effects of the diseases, the etiologic agent(s) of both CSD and BA have evaded identification. This invention describes the identification of an organism, named *R. henselae* herein, which is causative of both diseases.

R. quintana has been associated with varied clinical syndromes including persistent fever with bacteremia in normal and immunosuppressed individuals (18, 23, 30, 34). Despite the association of *R. quintana* with disease, *R. quintana*, has not been firmly linked to CSD. Given the controversy surrounding the etiology of CSD and the association *R. quintana* with human disease, there exists a need for a method of directly detecting each of these organisms in lymph node tissue from CSD patients.

The invention meets this need by providing a nucleic acid based method of detecting *R. quintana* infection in a subject. The isolated nucleic acid sequences of the present invention allow primers and probes to be readily designed for such a nucleic acid based detection system.

A need also exists to rapidly identify the presence of infection of a patient by *R. henselae*. Rapid and efficient determination of such infections will aid in the diagnosis of the previously ambiguous symptoms associated

with infection by *R. henselae* and therefore facilitate the evaluation of a proper course of treatment.

The present invention meets this need by providing
5 purified antigenic polypeptides necessary for detecting
the presence of *R. henselae* antibodies circulating in the
serum of patients presently, or previously infected with
R. henselae. These same purified antigenic polypeptides
can be used to produce antibodies which themselves may be
10 utilized in a method to detect the presence of *R. henselae*
antigen present in a subject, and therefore determine
whether a subject is currently, or has previously been
infected with *R. henselae*.

15

SUMMARY OF THE INVENTION

The present invention relates to a method of
diagnosing cat scratch disease and a method of diagnosing
bacillary angiomatosis in a subject by detecting the
20 presence of *Rochalimaea henselae* or an immunogenically
specific determinant thereof in the subject. Also
provided by the present invention is a method of
diagnosing cat scratch disease and a method of diagnosing
bacillary angiomatosis in a subject by detecting the
25 presence of antibodies in a subject which bind to
antigenic determinants of *R. henselae*.

30

The present invention also provides isolated nucleic
acids encoding immunogenic polypeptides of *R. henselae*.
35 Vectors are also provided comprising the nucleic
acids of the present invention. The vectors can be used
in a host expression system to produce antigenic
polypeptides reagents for diagnostic and prophylactic
applications.

The present invention further relates to a method of diagnosing *Rochalimaea quintana* infection in a subject by detecting the presence of a nucleic acid specific to *Rochalimaea quintana* in a sample from the subject.

5

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the number of comigrating DNA fragments and the estimated percentage of sequence divergence among organisms related to *R. henselae*. Numbers in parentheses (along the diagonal) indicate the total number of fragments used in analysis of each species. Fractions in the upper right sector indicate the number of comigrating DNA fragments for each pair of species divided by the number of fragments present for both species. Numbers in the lower left sector correspond to the estimated percentage of sequence divergences.

Figure 2 shows the distribution of *R. henselae* specific antibody titers among persons diagnosed with cat scratch disease syndrome.

Figure 3 shows the distribution of *R. henselae* specific antibody titers among healthy persons.

Figure 4 shows the nucleotide sequence alignment for the three regions of the antigen gene corresponding to primers CAT1 and CAT2 and oligonucleotide probes RH1 and RQ1. The antigen gene sequences were aligned for maximal homology using only the portion corresponding to the primer and probe sequences. The sequences for *R. henselae* and corresponding base substitutions (from the *R. henselae* sequence) for other species are shown, and conserved positions are indicated with a period. The complement of PCR primer CAT2 is shown [CAT2 (C)].

DETAILED DESCRIPTION OF THE INVENTION

Purified *R. henselae* Antigen

- 5 The invention provides immunogenically specific proteins or antigenic polypeptide fragments of *R. henselae*. These immunogenically specific proteins or polypeptides are those that specifically bind antibodies that specifically bind *R. henselae*. The immunogenically
- 10 specific proteins or polypeptide fragments of *R. quintana* are those that specifically bind antibodies that specifically bind *R. quintana*. Specific binding denotes the absence of cross reactivity with antibodies or antigens from organisms other than the specified species.
- 15 As contemplated herein, an antigen or antibody that is specific for both *R. henselae* and *R. quintana* can bind antibodies or antigens from both of these bacteria, but not antibodies or antigens from other organisms.
- 20 An immunogenically specific determinant of *R. henselae* or *R. quintana* can be isolated from the whole organism by chemical or mechanical disruption of the organism. For example, a carbohydrate moiety of the organism can be obtained by standard methods such as
- 25 digesting the organism with a protease to remove protein moieties.
- Alternatively, a protein moiety of *R. henselae* or *R. quintana* can be obtained by treating the whole organism
- 30 with an ionic detergent such as sodium dodecyl sulfate or a nonionic detergent such as Triton X-100 ($C_{12}H_{25}NO_{11}$ average) or ethylphenyl-polyethylene glycol (NP-40, Shell Oil Company). The protein fragments so obtained can be tested for immunogenicity and specificity as described above.
- 35 Other immunogenically specific determinants of the organism can be obtained by the standard methods described above.

Immunogenically specific determinants or antigenic polypeptides of this invention can be obtained by synthesizing a vector comprising a nucleic acid sequence encoding an immunogenically specific determinant of *R. henselae* or *R. quintana*. Examples of nucleic acids that encode immunogenic determinants of and *R. henselae* are provided herein, specifically in Examples 3 and 4. The vector can then be placed in a host wherein the immunogenically specific determinant can be synthesized.

10 The selection of a nucleic acid sequence that encodes an immunogenically specific determinant can be accomplished by screening clone libraries of the organism's DNA. Briefly, the bacteria are lysed and the DNA extracted via standard procedure using 1% sodium dodecyl sulfate and

15 proteinase K. The resulting DNA is then partially digested with restriction endonuclease *EcoRI*, size fractionated and gel purified (agarose gel electrophoresis), and cloned into lambda phage vector lambda ZapII following standard procedures such as

20 described in Maniatis et al. (20). The recombinant plaques are screened for antigen production via ELISA with primary antibody being human or other non-human (e.g., feline) convalescent sera absorbed with an *E. coli* lysate. Alternatively, antigen produced by the recombinant clones

25 can be screened by expressing the antigen and incubating the antigen with antibody-containing serum obtained from patients diagnosed with cat scratch disease. Those recombinant clones expressing *R. henselae*-specific antigen can then be identified by conventional methods (14).

30 Antigen expressing clones are subsequently subcloned and sequenced. Probes can then be derived that are specific for each species.

The subclones expressing species-specific antigens

35 are sequenced and corresponding synthetic peptides can be constructed from the deduced amino acid sequence for use as diagnostic antigens or immunogens. Alternatively,

recombinant antigens synthesized from polypeptide-expressing vectors in an appropriate host cell line could be purified by affinity chromatography, polyacrylamide gel electrophoresis, fast peptide liquid chromatography
5 (FPLC), high pressure liquid chromatography, and the like.

For example, the present invention provides an isolated nucleic acid as set forth in the Sequence Listing as SEQ ID NO:11 which encodes a polypeptide with an open
10 reading frame of 148 amino acids with an approximate molecular weight of 17-kDa (17-kilodaltons). As shown in Example 4, this purified polypeptide specifically binds to antibody present in the serum of patients diagnosed with cat scratch disease. This isolated polypeptide,
15 therefore, provides a valuable reagent for diagnostic applications.

Similarly, the present invention also provides an isolated nucleic acid as set forth in the Sequence Listing
20 as SEQ ID NO:7. The polypeptide encoded by this isolated nucleic acid has an open reading frame of 503 amino acids with an approximate molecular weight of 60-kDa (60-kilodaltons) and represents a heat-shock protein encoded by the htrA gene of *R. henselae*. As shown in Example 3,
25 this isolated nucleic acid provides a diagnostic reagent which is valuable in nucleic acid-based detection assays of *R. henselae* infection.

Once the amino acid sequence of the antigen is
30 provided, it is also possible to synthesize, using standard peptide synthesis techniques, peptide fragments homologous to immunoreactive regions of the antigen and test these fragments for their antigenicity using conventional techniques (14). These antigenic fragments
35 can also be modified by inclusion, deletion, or modification of particular amino acid residues in the derived sequences. Thus, synthesis or purification of an

extremely large number of peptides derived from the antigens provided is possible.

Similarly, fragments of the antigenic polypeptides provided can be cloned into peptide expression vectors for peptide synthesis *in vivo*. Such *in vivo* synthesized polypeptides can be readily purified using techniques such as affinity chromatography.

The amino acid sequences of the present polypeptides can contain an immunoreactive portion of the antigen attached to sequences designed to provide for some additional property, such as solubility. The amino acid sequences of the polypeptides can also include sequences in which one or more amino acids have been substituted with another amino acid to provide for some additional property, such as to remove/add amino acids capable of disulfide bonding, to increase its bio-longevity, alter enzymatic activity, or alter interactions with gastric acidity. In any case, the peptide must possess a bioactive property, such as immunoreactivity, immunogenicity, etc.

A nonpathogenic *R. henselae* or *R. quintana* antigen can be derived by modifying the organism using standard techniques. For example, the whole cell antigen can be subjected to gamma irradiation to render the organism nonpathogenic. Other standard methods of inactivating whole cell antigen include treatment with β -propiolactone or formalin (14).

Determining Antigenicity and Specificity

The antigenicity and specificity of the immunogenic fragments (antigenic polypeptides) or modified whole bacteria can be determined by the usual methods. Briefly, various concentrations of a putative inactivated

(nonpathogenic) immunogenically specific determinant are prepared and administered to an animal and the immunological response (i.e., the production of antibodies) of an animal to each concentration is
5 determined. The amounts of antigen or inactivated or modified-live organism administered depends on the subject, e.g. a human or a cat, the condition of the subject, the size of the subject, etc. Thereafter an animal so inoculated to the nonpathogenic antigen can be
10 exposed to the pathogenic organism to test the potential vaccine effect of the immunogenically specific determinant. The specificity of a putative immunogenically specific determinant can be ascertained by testing sera or other fluid from the inoculated animal for
15 cross reactivity with another species (14).

Serological Diagnosis

The present invention provides a method of diagnosing
20 cat scratch disease in a subject comprising detecting the presence of *Rochalimaea henselae* or an immunogenically specific determinant thereof (hereinafter collectively referred to as "*R. henselae* antigen") in the subject. The subject can be a human or other animal. As used herein,
25 an "immunogenically specific determinant" can be on an intact *R. henselae* or an antigenic fragment of *R. henselae*.

Given the subject discovery that the presence of *R.*
30 *henselae* is associated with cat scratch disease, bacillary angiomatosis and splenic hepatic peliosis, many well-known methods of detecting a bacteria can be applied to detect *R. henselae* and diagnose a disease. In one example of the method of diagnosing cat scratch disease, the step of
35 detecting *R. henselae* antigen is performed by contacting a fluid or tissue sample from the subject with an amount of a purified ligand, e.g. antibodies or antibody fragments,

that specifically bind *R. henselae* antigen and detecting the reaction of the ligand with *R. henselae* antigen. As contemplated herein, the term "antibody" includes an intact antibody, a fragment of an antibody or another
5 reagent (ligand) that binds nonrandomly with the antigen. The fluid sample of this method can comprise any body fluid which would contain *R. henselae*; for example, blood, plasma and serum. Other possible examples of body fluids include urine, sputum, mucus and the like.

10

In an alternative embodiment, the method of diagnosing cat scratch disease of the present invention can be such that the presence of *R. henselae* is determined by detecting the presence of an antibody from the subject
15 which specifically binds with *R. henselae* antigen. The presence of antibody which specifically binds with *R. henselae* indicates the presence of infection by *R. henselae*. As used herein, the term "specifically binds" denotes an antibody or other ligand that does not cross
20 react, or bind, substantially with any antigen other than the one specified, in this case, *R. henselae* antigen.

When the method of diagnosing cat scratch disease is by detecting the presence of an antibody specifically
25 reactive (i.e. specifically binds) with *R. henselae* antigen, the step of detecting the presence of an antibody specifically reactive to *R. henselae* antigen can, for example, include the steps of contacting a fluid or tissue sample from the subject with an amount of *R. henselae*
30 antigen that binds an antibody which specifically binds with *R. henselae* and detecting the binding of the *R. henselae* antigen with the antibody. It is expected that the antigen used will specifically bind antibodies to *R. henselae* produced in the course of *R. henselae* infection.
35 One method of conducting such a diagnosis is illustrated in Example 2.

Detecting the reaction of the ligand with *R. henselae* antigen can be facilitated by the use of a ligand that is bound to a detectable moiety. Such a detectable moiety will allow visual detection of a precipitate or a color change, visual detection by microscopy, or automated detection by spectrometry or radiometric measurement or the like. Examples of detectable moieties include fluorescein and rhodamine (for fluorescence microscopy), horseradish peroxidase (for either light microscopy or electron microscopy and biochemical detection), biotin-streptavidin (for light or electron microscopy) and alkaline phosphatase (for biochemical detection by color change). The detection method and detectable moiety used can be selected from the list above or other suitable examples by the standard criteria applied to such selections (14).

In the diagnostic methods of the present invention, the step of detecting the reaction of the ligand with *R. henselae* antigen can be further aided, in appropriate instances, by the use of a secondary antibody or other ligand which binds, either specifically with a different epitope or nonspecifically with the ligand or bound antibody.

In the diagnostic method which detects the presence of an antibody which specifically binds with *R. henselae* antigen, the *R. henselae* antigen can be bound to a substrate and contacted by a fluid sample such as blood, plasma or serum. This sample can be taken directly from the patient or in a partially purified form. In this manner, antibodies specific for *R. henselae* antigen (the primary antibody) will specifically bind with the bound *R. henselae* antigen. Thereafter, a secondary antibody bound to, or labeled with, a detectable moiety can be added to enhance the detection of the primary antibody. Generally, the secondary antibody will be selected for its ability to

bind with multiple sites on the primary antibody. Thus, for example, several molecules of the secondary antibody can bind with each primary antibody, making the primary antibody more detectable.

5

Detecting methods such as immunofluorescence assays (IFA) and enzyme linked immunosorbent assays (ELISA) can be readily adapted to accomplish the detection of both *R. henselae* antigen and antibodies which specifically bind therewith. An example of an IFA protocol is provided in Example 2. The indirect immunocytochemical methods taught in Example 2 will be generally applicable for the detection of antigens or antibodies specific to an organism. An ELISA method effective for the diagnosis of cat scratch disease based on the detection of human IgG antibodies can, for example, be as follows: (1) bind the antigen (*R. henselae* antigen) to a substrate; (2) contact the bound antigen with a serum sample, containing antibodies reactive with *R. henselae* antigen, from a subject; (3) contact the above with an anti-human IgG antibody (secondary antibody) bound to a detectable moiety (e.g., horseradish peroxidase enzyme or alkaline phosphatase enzyme); (4) contact the above with the substrate for the enzyme; (5) contact the above with a color reagent; (6) observe color change in the presence of IgG antibody which specifically binds with *R. henselae* antigen. An indirect enzyme-linked immunosorbent assay (ELISA) for IgG antibodies against *R. henselae* is briefly as follows: Flat-bottomed 96-well polystyrene plates are coated with *R. henselae* or negative control antigen and allowed to incubate overnight. The next day, two-fold serial dilutions of test sera and 5 negative control sera, mouse anti-human IgG conjugated to horseradish peroxidase, and finally the substrate ABTS (2, 2'-azino-di-[3-ethyl-benzothiazoline sulfonate]) are added to each well sequentially. Between each step, plates are incubated for 1 hour at 37°C, and then washed 3 times with 0.1% Tween 20

in phosphate-buff red saline (pH 7.4). Dilutions of sera
ar considered positive when the difference in absorbance
between that serum specimen when tested with *R. henselae*
antigen and the negative control antigen exceeds the mean
5 plus 3 standard deviations of the 5 negative control sera
tested with both *R. henselae* and negative control
antigens.

A modification of the above ELISA effective for
10 diagnosis of cat scratch disease and bacillary
angiomatosis based on the detection of human IgM
antibodies can be as follows: (1) bind an anti-human IgM
antibody capable of reacting with a human IgM antibody to
a substrate (antibody capture); (2) contact the bound
15 antibody with a serum sample from a subject; (3) contact
the above with *R. henselae* antigen; (4) contact the above
with a rabbit anti-*R. henselae* antibody; (5) contact the
above with an anti-rabbit antibody bound to a detectable
moiety (e.g., horseradish peroxidase enzyme); (6) contact
20 the above with substrate for the enzyme; (7) contact the
above with a color reagent; (8) observe a color change in
the presence of an IgM antibody specifically reactive with
R. henselae antigen. For the IgM capture ELISA, flat-
bottomed 96-well polystyrene plates are coated with goat
25 anti-human IgM antibody, followed by serial two-fold
dilutions of sera including 5 negative controls, *R.*
henselae or negative control antigens, *R. henselae*
hyperimmune rabbit antisera, and goat anti-rabbit
conjugated to horseradish peroxidase and the substrate
30 (ABTS). Between each step, plates are incubated for 1
hour at 37°C, and then washed 3 times with 0.1% Tween 20
in phosphate-buffered saline (pH 7.4). Dilutions of sera
are considered positive when the difference in absorbance
between that serum specimen when tested with *R. henselae*
35 antigen and the negative control antigen exceeds the mean
plus 3 standard deviations of the 5 negative control sera

tested with both *R. henselae* and negative control antigens.

Alternatively, methods such as immunoblot analysis can be readily adapted to detect the presence of *R.*

- 5 *henselae* antigen and antibodies which specifically bind therewith. An example of an immunoblot analysis is provided in Example 4. The immunoblot analysis taught in Example 4 can be applicable for the detection of antigens or antibodies specific to the whole organism, or to
- 10 antigens or antibodies specific to only a fragment of the whole organism. An immunoblot analysis effective for the detection of *R. henselae* antigen or antibodies specific to *R. henselae* can, for example, be as follows: (1) grow recombinant vectors containing *R. henselae* encoding
- 15 nucleic acids in an appropriate host for expression of the recombinant nucleic acid; (2) induce the host to express the recombinant nucleic acid; (3) solubilize the host to release the polypeptide expressed by the vector; (4) electrophorese the released polypeptides on sodium dodecyl
- 20 sulfate-polyacrylamide gels; (5) transfer the proteins to nitrocellulose membranes; (6) incubate the nitrocellulose membranes with serum from patients potentially infected with *R. henselae*; and (7) detecting the bound antigen or antibody by reacting the filters with goat anti-human IgG
- 25 conjugated with horseradish peroxidase.

- Another immunologic technique that can be useful in the detection of *R. henselae* infection utilizes monoclonal antibodies for detection of antibodies which specifically
- 30 bind with *R. henselae* antigen. Briefly, sera from the subject is incubated with *R. henselae* antigen bound to a substrate (e.g. an ELISA 96-well plate). Excess sera is thoroughly washed away. A labeled (enzyme-linked, fluorescent, radioactive, etc.) monoclonal antibody is
- 35 then incubated with the previously reacted antigen-serum antibody complex. The amount of inhibition of monoclonal antibody binding is measured relative to a control (no

patient serum antibody). The degree of monoclonal antibody inhibition is a very specific test for a particular species since it is based on monoclonal antibody binding specificity.

5

A micro-agglutination test can also be used to detect the presence of *R. henselae* in a subject. Briefly, latex beads (or red blood cells) are coated with *R. henselae* antigen and mixed with serum from the subject, such that
10 antibodies in the tissue or body fluids that specifically bind with *R. henselae* antigen crosslink with the antigen, causing agglutination. The agglutinated antigen-antibody complexes form a precipitate, visible with the naked eye. In a modification of the above test, antibodies which
15 specifically reactive bind *R. henselae* antigen can be bound to the beads and antigen in the serum thereby detected. Other fluids of a subject can be effectively used.

20 In addition, as in a typical sandwich assay, the antibody is bound to a substrate and incubated with an *R. henselae* antigen. Thereafter, a secondary labeled antibody is bound to epitopes not recognized by the first antibody and the secondary antibody is detected.

25

The specific reagents and protocols for use in the detection methods described above and similar indirect immunocytochemical methods can be selected from those available in the art based on standard criteria (14).

30

The instant invention also provides a method of diagnosing clinical bacillary angiomatosis in a subject by detecting the presence of *R. henselae* antigen in the subject. The step of detecting the presence of *R.*
35 *henselae* can be accomplished using the same protocols as taught above for the diagnosis of cat scratch disease.

Because *R. quintana* is also associated with BA, the instant invention also provides a method of diagnosing clinical bacillary angiomatosis in a subject by detecting the presence of *R. quintana* antigen in the subject. The
5 step of detecting the presence of *R. quintana* can be accomplished using the same protocols as taught above for the diagnosis of cat scratch disease.

10 Nucleic Acids and Nucleic Acid-Based Diagnosis

In the diagnostic methods of the instant invention, the presence of *R. henselae* can also be determined by detecting the presence of a nucleic acid sequence specific
15 for *R. henselae*. Thus, CSD can be diagnosed by detecting in a patient sample a nucleic acid that is specific for *R. henselae*. The nucleic acid can be detected by detecting the presence of an amplification product following
polymerase chain reaction (PCR), or other routine
20 amplification method, using species-specific primers. Alternatively, the nucleic acid can be detected by probing non-specific amplification products of PCR with a species-specific probe, as illustrated in Example 3.
Additionally, a species-specific probe can be used in an
25 *in situ* hybridization protocol to detect the presence of a nucleic acid sequence specific for the organism, for example in lymph node biopsy tissue from a patient suspected of having BA or CSD.

30 The invention also provides a method of diagnosing current or previous *R. quintana* infection in a subject by detecting the presence of a nucleic acid sequence specific for *R. quintana* by routine methods as described herein. By detecting *R. quintana*, bacillary angiomatosis can be
35 diagnosed, because *R. quintana* is associated with BA (15).

A rapid two step method of diagnosing cat scratch disease or bacillary angiomatosis in a subject is provided. The method comprises amplifying DNA from the subject using a primer mixture consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4. Following amplification, CSD or BA or both can be diagnosed by contacting the amplified DNA with a probe consisting of the nucleic acid of SEQ ID NO:5 and detecting the hybridization of the probe with the amplified DNA, the existence of hybridization indicating the presence of *R. henselae*, which is correlated with cat scratch disease, and contacting the amplified DNA with a probe consisting of the nucleic acid of SEQ ID NO:6 and detecting the hybridization of the probe with the amplified DNA, the existence of hybridization indicating the presence of *R. quintana*, which is correlated with bacillary angiomatosis. The steps of one example of this method are set out in detail in Example 3.

As more specifically exemplified below, a nucleic acid sequence specific for *R. henselae* can comprise nucleic acids coding for 16S ribosomal RNA subunit. Alternatively, a nucleic acid sequence specific for *R. henselae* can comprise nucleic acids coding for citrate synthase. It is apparent that a skilled artisan can apply the methods described herein for detecting the citrate synthase gene and the 16S ribosomal RNA gene to detect other nucleic acid sequences specific for *R. henselae*. Examples of other sequences specific for *R. henselae* can include the genes for heat shock protein, other antigenic proteins and certain metabolic and synthetic enzymes. The specificity of these sequences for *R. henselae* can be determined by conducting a computerized comparison with known sequences, catalogued in GenBank, a computerized database, using, for example, the computer program Gap of the Genetics Computer Group, which searches the catalogued sequences for similarities to the gene in question.

Example 3 describes examples of nucleic acids specific for *R. henselae* and *R. quintana*. For example, a nucleic acid specific for *R. henselae* consists of the nucleotides in the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:7. This gene, *htrA*, encodes the antigenic heat shock protein of *R. henselae*. A nucleic acid consisting of the nucleotides in the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:5 is derived from SEQ ID NO:7, and is also specific for *R. henselae* (SEQ ID NO:8).

A nucleic acid specific for *R. quintana*, comprising the nucleotides in the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:6 is provided. This nucleic acid is effective as a species specific probe for detecting *R. quintana* infection as shown in Example 3. Having provided the partial nucleotide sequence for the *R. quintana htrA* gene, the remainder of the sequence can be readily obtained using standard methods, such as those described in Example 1 and elsewhere herein. Additionally, given the present invention's teaching of the sequence of the *htrA* gene for *R. henselae*, other sequences in the corresponding *R. quintana htrA* gene can be routinely determined to be specific for *R. quintana* merely by obtaining the full sequence and testing segments for specificity in the methods taught in Example 3.

Example 4 describes another example of a nucleic acid specific for *R. henselae*, consisting of the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:11. Immunoanalysis of this 17-kDa polypeptide suggests that this polypeptide is the immunodominant antigen of *R. henselae*, and is therefore a valuable immuno-reagent which can be used in diagnostic and prophylactic applications. Given the discovery of the nucleic acid sequence of the 17-kDa polypeptide, a skilled artisan in the relevant field can readily appreciate that nucleic acid reagents,

such as primers and probes, can easily be designed for use in a nucleic acid detection system to detect the presence of a nucleic acid encoding the 17-kDa protein.

5 By "isolated nucleic acid" is meant the nucleic acid is separated from at least some of other components of the naturally occurring organism, for example, the cell structural components. The isolation of the nucleic acids can therefore be accomplished by techniques such as cell
10 lysis followed by phenol plus chloroform extraction, followed by ethanol precipitation of the nucleic acids (20). It is not contemplated that the isolated nucleic acids are necessarily totally pure of non-nucleic acid components, but that the isolated nucleic acids are
15 isolated to a degree of purification to be used in a clinical, diagnostic, experimental, other procedure such as gel electrophoresis, Southern or dot blot hybridization, or PCR. A skilled artisan in the field will readily appreciate that there are a multitude of
20 procedures which may be used to isolate the nucleic acids prior to their use in other procedures. These include, but are not limited to, lysis of the cell followed by gel filtration or anion exchange chromatography, binding DNA to silica in the form of glass beads, filters or diatoms
25 in the presence of high concentration of chaotropic salts, or ethanol precipitation of the nucleic acids.

The nucleic acids of the present invention can include positive and negative strand RNA as well as DNA
30 and is meant to include genomic and subgenomic nucleic acids found in the naturally occurring organism. The nucleic acids contemplated by the present invention include double stranded and single stranded DNA of the genome, complementary positive stranded cRNA and mRNA, and
35 complementary cDNA produced therefrom and any nucleic acid which can selectively or specifically hybridize to or encode the isolated nucleic acids provided herein.

The present invention provides isolated nucleic acids that can selectively hybridize with and be used to either detect the presence of, or amplify nucleic acids comprising the nucleotide sequences set forth in the sequence listing as SEQ ID NO:7 and SEQ ID NO:11. The stringency conditions can be those typically used in PCR protocols. In particular, an isolated nucleic acid that hybridizes with (or amplifies) the nucleic acids set forth in SEQ ID NO:7 and SEQ ID NO:11 under high stringency conditions and has at least 70% complementarity with the segment of the nucleic acid of SEQ ID NO:7 and SEQ ID NO:11 to which it hybridizes is also provided. The selectively hybridizing nucleic acids can be used, for example, as probes or primers for detecting the presence of *R. henselae* or a genetic homolog thereof that has the nucleic acid to which the primer or probe hybridizes. Thus, the invention provides a method of detecting *R. henselae* and genetic homologs thereof in a specimen and thereby detecting infection in a subject, comprising detecting the presence of a selectively hybridizing nucleic acid in a specimen from the subject, the presence of the nucleic acid indicating infection with *R. henselae* or a genetic homolog.

Also provided is a nucleic acid that selectively or specifically hybridizes with the nucleic acid of SEQ ID NO:7 under high stringency conditions and has about 85% sequence complementarity with the segment to which it hybridizes. As shown in Example 3, the *htrA* gene sequences of the four related *Rochalimaea* species demonstrate from about 85% to about 92% overall sequence identity with *R. quintana* being the most similar.

The invention also provides for a nucleic acid of at least 15 nucleotides in length which selectively hybridizes under polymerase chain reaction (PCR) conditions to the nucleic acid set forth in the Sequenc

Listing as SEQ ID NO:11. Since PCR is used extensively in the art, the conditions for PCR are widely known in the art and will readily be apparent to a skilled practitioner. These conditions are stated in instructions that accompany PCR machines, in standardized PCR kits provided by many biochemical reagent suppliers, as well as in many articles and standard texts.

The present invention also provides isolated nucleic acid of at least 15 nucleotides in length which specifically hybridizes with the nucleic acid set forth in the Sequence Listing as SEQ ID NO:11 under hybridization stringency conditions of 60°C and 5X SSC (1X SSC = 8.765 grams Sodium Chloride and 4.410 grams Sodium Citrate in a volume of 1 liter of H₂O, pH 7.0), followed by the initial washing stringency conditions of room temperature, 2X SSC and 0.1% SDS (sodium dodecyl sulfate), and two final washes under the stringency conditions of 50°C, 0.5% SSC and 0.1% SDS is provided.

20

The present invention therefore provides for a purified homolog of the polypeptides consisting of the polypeptides set forth in the Sequence Listing as SEQ ID NO:7 and SEQ ID NO:11. Such homolog may be obtained from other bacterial species whose genome encodes a homolog of the purified polypeptides of the present invention. For instance, Example 4 provides an immunoscreening assay in which a homolog of the 17-kDa protein of SEQ ID NO:11 can be detected. Methods used to isolate a nucleic acid encoding a bacterial or other homolog to SEQ ID NO:11 include, but are not limited to, screening the genome of a species believed to encode a homolog by nucleic acid hybridization methods or through polymerase chain reaction (PCR) techniques. Materials suitable for screening include, but are not limited to, cDNA or genomic libraries of the appropriate species cloned into lambda, cosmid,

yeast, mammalian, or plasmid cloning vectors, DNA isolated and subjected to Southern blot analysis, RNA isolated and subjected to Northern blot analysis, and isolated DNA or RNA used as a template for PCR.

5

Also as used herein to describe nucleic acids, the terms "selectively hybridizes" and "specifically hybridizes" exclude the occasional randomly hybridizing nucleic acids as well as nucleic acids that encode heat shock proteins from other genera. The hybridizing nucleic acids can be used, for example, as probes or primers for detecting the presence of and location of a gene encoding a protein of the invention. The hybridizing nucleic acid can encode a polypeptide, and can, thereby, be placed in a suitable vector and host to produce the antigen, a functionally similar antigen, or an antigenic polypeptide fragment.

The selectively hybridizing nucleic acids of the invention can have at least 80%, 85%, 90%, 95%, 97%, 98% and 99% complementarity with the segment and strand of the sequence to which it hybridizes. The nucleic acids can be at least 12 and up to 4000 nucleotides in length. Thus, the nucleic acid can be an alternative coding sequence for the antigen, or can be used as a probe or primer for detecting the presence of the nucleic acid encoding the antigen. If used as primers, the invention provides compositions including at least two nucleic acids which selectively hybridize with different regions of a nucleic acid so as to amplify a desired region. For the purpose of detecting the presence of the species-specific antigen-encoding gene, the degree of complementarity between the hybridizing nucleic acid (probe or primer) and the sequence to which it hybridizes (DNA from a sample) should be at least enough, and the sequence should be long

enough, to exclude random hybridization with a nucleic acid of another species or an unrelated protein. Figure 4 illustrates the relationship between complementarity and probe length.

5

The selectively hybridizing nucleic acid can also be selective for the genus, *Rochalimaea*, or a subset of species in the genus. For example, the primers CAT1 and CAT2 selectively amplify a product from both *R. quintana* and *R. henselae*, which can then be probed with a species specific nucleic acid. The invention provides examples of a range of selectively hybridizing nucleic acids, so that the degree of complementarity required to distinguish selectively hybridizing from nonselectively hybridizing nucleic acids under stringent conditions can be clearly determined for each nucleic acid.

"High stringency conditions" refers to the washing conditions used in a hybridization protocol. In general, the washing conditions should be a combination of temperature and salt concentration chosen so that the denaturation temperature is approximately 5-20°C below the calculated T_m of the hybrid under study. The temperature and salt conditions are readily determined empirically in preliminary experiments in which samples of reference DNA immobilized on filters are hybridized to the probe or protein coding nucleic acid of interest and then washed under conditions of different stringencies. For example, high stringency conditions for the present selectively hybridizing nucleic acids are given in Example 3. The specific stringency conditions are readily tested and the parameters altered are readily apparent to one skilled in the art. For example, $MgCl_2$ concentrations used in the reaction buffer can be altered to increase the specificity with which the primer binds to the template, but the

concentration range of this compound used in hybridization reactions is narrow, and therefore, the proper stringency level is easily determined. For example, hybridizations with oligonucleotide probes 18 nucleotides in length can be done at 5-10°C below the estimated T_m in 6X SSPE, then washed at the same temperature in 2X SSPE (29). The T_m of such an oligonucleotide can be estimated by allowing 2°C for each A or T nucleotide, and 4°C for each G or C. An 18 nucleotide probe of 50% G+C would, therefore, have an approximate T_m of 54°C. Likewise, the starting salt concentration of an 18 nucleotide primer or probe would be about 100-200mM. Thus, stringent conditions for such an 18 nucleotide primer or probe would be a T_m of about 54°C and a starting salt concentration of about 150mM and modified accordingly by preliminary experiments. T_m values can also be calculated for a variety of conditions utilizing commercially available computer software (e.g., OLIGOTM).

20 Additionally, the nucleic acids of the invention can have at least 80% homology with the coding nucleotides of SEQ ID NO:7 and SEQ ID NO:11 that are not subject to the degeneracy of the genetic code, i.e., with the non-"wobble" nucleotides (the wobble nucleotides usually being the third nucleotide in a codon) in the coding sequence. Preferably, the nucleic acids will have 90%, or more preferably, 95%, or even more preferably, 99% homology with the coding nucleotides of SEQ ID NO:7 and SEQ ID NO:11 that are not subject to the degeneracy of the genetic code.

Modifications to the nucleic acids of the invention are also contemplated as long as the essential structure and function of the polypeptide encoded by the nucleic acids is maintained. Likewise, fragments used as primers or

probes can have substitutions so long as enough complementary bases exist for selective amplification.

One skilled in the art can readily obtain the nucleic
5 acids of the present invention using routine methods to
synthesize a full gene as well as shorter nucleotide
fragments. For example, techniques for obtaining nucleic
acids such as those provided in the Sequence Listing are
specifically provided in the application. Furthermore,
10 additional methods are provided in the art that can be
utilized without significant modification. Ferretti et
al. (38) and Wosnick et al. (39) show routine methods to
synthesize a gene of known sequence. More specifically,
Ferretti et al. teach the synthesis of a 1057 base pair
15 synthetic bovine rhodopsin gene from synthetic
oligonucleotides. The synthesized gene was faithful to
the known sequence (first sentence, page 603),
demonstrating the reliability of this method of gene
synthesis. Additionally, Wosnick et al. teach the
20 synthesis of a maize glutathione-transferase (GST) gene
using an efficient, one-step annealing/ligation protocol.
This technique also produced a complete synthetic gene
with 100% fidelity, which demonstrates the routine nature
of this protocol.

25

As described herein, the nucleic acids can be expressed
to provide antigenic polypeptides of the invention.

Diagnostic Kits

30

The present invention further provides a kit for the
diagnosis of cat scratch disease. Such a kit can be an
ELISA kit and can comprise the substrate, antigen, primary
and secondary antibodies when appropriate, and any other
35 necessary reagents such as detectable moieties, enzyme

substrates and color reagents as described above. The diagnostic kit of the present invention can alternatively be constructed to detect nucleic acid sequences specific for *R. henselae* antigen comprising the standard kit

5 components such as the substrate and reagents such as those set forth in Example 1 for the detection of nucleic acid sequences. The diagnostic kit can, alternatively, be an IFA kit generally comprising the components and reagents described in Example 2 below. Because *R.*

10 *henselae* infection can be diagnosed by detecting nucleic acids specific for *R. henselae* in tissue and body fluids such as blood and serum, it will be apparent to an artisan that a kit can be constructed that utilizes the nucleic acid detection methods taught herein. It is contemplated

15 that the diagnostic kits will further comprise a positive and negative control test.

The particular reagents and other components included in the diagnostic kits of the present invention can be

20 selected from those available in the art in accord with the specific diagnostic method practiced in the kit. Such kits can be used to detect *R. henselae* antigen and antibodies which specifically bind therewith in tissue and fluid samples from a subject and in cultures of

25 microorganisms obtained from the tissue or fluids of a subject.

The kits of the instant invention can also be used in a method of diagnosing bacillary angiomatosis.

30

Vaccines

Also provided by the present invention is a vaccine comprising an immunogenic amount of a nonpathogenic

35 *Rochalimaea henselae* or an immunogenically specific

determinant thereof and a pharmaceutically acceptable carrier. Alternatively, the vaccine can comprise an antigenic polypeptide that specifically binds antibodies that specifically bind both *R. henselae* and *R. quintana* and a pharmaceutically acceptable carrier.

The nonpathogenic *R. henselae* antigen of this invention can be used in the construction of a vaccine comprising an immunogenic amount of *R. henselae* antigen and a pharmaceutically acceptable carrier. This *R. henselae* antigen can be killed, modified live or immunogenic fragments (antigenic polypeptides) of *R. henselae*. Alternatively, mixtures of intact *R. henselae* and immunogenic fragments can be used. The vaccine can then be used in a method of preventing cat scratch disease in a subject by administering the vaccine to the subject. The vaccine can also be used in a method of preventing bacillary angiomatosis in a subject by administering the vaccine to the subject. Furthermore, the fact that other disease syndromes are associated with *R. henselae* infection, means that such diseases can also be prevented by use of the vaccines of this invention. The prevention methods will work when the subject is a human, or likewise when the subject is a nonhuman animal, such as a cat.

For example, the vaccine can comprise an antigenic protein encoded by the nucleic acid of SEQ ID NO:7. This protein (SEQ ID NOs:7 and 8) is the *R. henselae* heat shock protein. The present purified heat shock protein strongly binds antibodies in rabbit serum raised against whole dead *R. henselae*. The homologous protein from *R. quintana* can also be the basis of a vaccine. To further elaborate the use of the antigen in a vaccine, standard methods can be used as described below to determine immunogenicity and immunogenic amounts.

The vaccine can also comprise the antigenic protein encoded by the nucleic acid of SEQ ID NO:11. Example 4 provides evidence that the 17-kDa protein encoded by the isolated nucleic acid of SEQ ID NO:11 is the major
5 immunodominant antigen of *R. henselae* and this protein therefore provides a candidate for development of a successful vaccine for protection against *R. henselae* infection.

10 The vaccine can also comprise an antigenic polypeptide fragment encoded by a nucleic acid that selectively hybridizes with the nucleic acids of SEQ ID NO:7 or SEQ ID NO:11 under high stringency conditions and is specific for *R. henselae*. Because the sequences of the *R. henselae* and
15 the *R. quintana htrA* genes share regions of high sequence similarity, a polypeptide encoded by those regions can be specific for both species and can be used in the vaccine against both CSD and BA.

20 The pharmaceutically acceptable carrier in the vaccine of the instant invention can comprise saline or other suitable carriers (1). An adjuvant can also be a part of the carrier of the vaccine, in which case it can be selected by standard criteria based on the particular *R.*
25 *henselae* antigen used, the mode of administration and the subject (2). Methods of administration can be by oral or sublingual means, or by injection, depending on the particular vaccine used and the subject to whom it is administered.

30

It can be appreciated from the above that the vaccine can be used as a prophylactic or a therapeutic. Thus, subjects with the disease can be treated utilizing the vaccine. Further, through such vaccination the spread of
35 disease between animals and humans can be prevented. For

example, a cat or dog can be immunized, thereby preventing much of the exposure risk to humans.

Immunogenic amounts of *R. henselae* antigen can be
5 determined using standard procedures. Briefly, various concentrations of a putative inactivated (nonpathogenic) immunogenically specific determinant are prepared, administered to an animal and the immunological response (i.e., the production of antibodies) of an animal to each
10 concentration is determined.

Thus, the invention provides methods of preventing or treating an *R. henselae* infection and the associated disease by administering the vaccine to a subject.
15

Other compositions of this invention include a purified *R. henselae* bound to a ligand, e.g. an antibody. The term "purified" is used herein to describe antigens, antibodies and other ligands that are substantially free of other
20 components of serum, blood or other body fluids, or other proteins associated with *R. henselae in vivo*.

A purified *R. henselae* antigen bound to a substrate and a ligand which specifically binds with *R. henselae* antigen
25 are also contemplated. Such a purified ligand which specifically binds with *R. henselae* antigen can be an antibody. The antibody can be a monoclonal antibody obtained by standard methods. The monoclonal antibody can be secreted by a hybridoma cell line specifically produced
30 for that purpose (14). Likewise, polyclonal antibodies which bind to *R. henselae* antigen are within the scope of the present invention. The polyclonal antibody can also be obtained by the standard immunization and purification protocols (14).

The antibody can be bound to a substrate or labeled with a detectable moiety or both bound and labeled. The detectable moieties contemplated with the composition of the present invention are those listed above in the
5 description of the diagnostic methods, including fluorescent, enzymatic, and radioactive markers.

The compositions of the instant application further include an antibody reactive to a unique portion of an
10 antibody which specifically binds with *R. henselae* antigen (primary antibody). The antibody which binds to the primary antibody is known as a secondary antibody, and can further comprise a detectable moiety. As described above, the binding of the secondary antibody to the primary
15 antibody which specifically binds with *R. henselae* antigen facilitates detection of the binding of primary antibody with *R. henselae* antigen.

An isolated immunogenically specific determinant or
20 fragment of *R. henselae* is also provided. The manner of obtaining such determinants is as described above for the construction of vaccines.

The following examples are intended to illustrate but
25 not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may be alternatively employed.

30

EXAMPLE 1

Identification of *R. henselae*

A previously asymptomatic HIV-antibody positive, 40-
35 year old man was admitted with a two month history of

daily fever, extreme fatigue, anorexia, and loss of 10 Kg of weight. Five weeks after admission, blood cultures taken on the first and eighth day of hospitalization were reported positive for a *Rochalimaea*-like organism. With
5 the presumptive diagnosis of trench fever, the patient was started on a 21-day course of doxycycline (100 mg, twice a day); after 48 hours he defervesced. Blood, urine, bone marrow, and bronchoalveolar lavage fluid cultures remained negative for mycobacteria and fungi. Six weeks after
10 discontinuation of therapy, fever, anorexia, and malaise recurred. Blood cultures drawn at this time were again positive for a *Rochalimaea*-like organism, and treatment with doxycycline for one month (same dose as above) was reinstituted with immediate and positive response. After
15 a second relapse of fever, the patient completed two months of doxycycline (same dose as above). Repeated blood cultures taken in the subsequent 6 months have been negative and symptoms associated with his initial infection have not recurred. The organism isolated is
20 hereinafter designated "Houston-1 isolate", which is considered the prototype isolate of *R. henselae*. The organism is deposited with the American Type Culture Collection (ATCC, Beltsville, MD) under Accession No. 49882.

25

A. Type Cultures

Two *Rochalimaea* isolates, representing two different recognized species, were obtained from the American Type
30 Culture Collection (ATCC, Beltsville, MD). *Rochalimaea quintana* (ATCC VR-358) and *R. vinsonii* (ATCC VR-152) were routinely cultivated at 35°C, 5% carbon dioxide atmosphere on tryptic soy agar, supplemented with 5% defibrinated sheep blood. *Rickettsia prowazekii*, isolate Breinl (ATCC
35 VR-142), was cultivated in Vero cell cultures and

cytoplasmic extracts containing rickettsiae were made by Regnery et al. (25).

B. Growth Characteristics

5

1. Isolation and cultivation of the organism from patient's blood (Houston-1 isolate).

Blood from the patient was drawn either directly into a
10 Wampole Isostat tube (Wampole Laboratories, Cranberry, N.J.) or simply into a Vacutainer tube containing EDTA (Becton Dickinson, Rutherford, N.J.); isolates were made using both starting preparations. The organism was reisolated from frozen (-85°C), EDTA treated blood without
15 significant loss of titer. Primary isolations were made on commercial brain heart infusion agar (BHIA) containing 5% sheep blood (BBL, Becton Dickinson, Cockeysville, Md.), tryptic soy agar (TSA) supplemented with 5% sheep blood (BBL), and heart infusion agar (HIA) containing 5% rabbit
20 blood (BBL). Cultures were maintained at 35°C in a humidified incubator containing 5% carbon dioxide. Bacteriological plates were routinely examined. As noted elsewhere, the Houston-1 isolate was cultivated from blood at various times during the course of the patients disease
25 episode including after relapse of fever following cessation of antibiotic therapy. The key to obtaining isolated cultures of *R. henselae* is to allow the culture to grow long enough for this slow-growing organism to form detectable colonies.

30

Blood from the febrile patient, when cultured on either commercial BHIA-sheep blood, TSA-sheep blood, or HIA-rabbit blood, yielded characteristic colonies which were visible after 9-10 days incubation. The approximate titer
35 of colony forming organisms in the patient's blood was 30

per milliliter after recrudescence of fever following the second course of antibiotic therapy. Primary colonies were deeply invaginated (cauliflower-like), firm, adherent, and tenaciously imbedded in the surface of the agar. All original individual colonies isolated from the patient's blood had similar morphologic and growth characteristics. Close inspection of subcultured plates revealed minute colony formation by 6 days after inoculation, although clear colony morphology was not evident at this time. After multiple passages of fresh colonies, incubation time to colony visualization decreased substantially and discrete colonies could be discerned after 3 to 4 days. The invaginated colony morphology became less pronounced after multiple, relatively rapid passages. Colony growth was not limited by incubation time, and colonies continued to grow progressively larger over a period of several weeks.

Several of these latter initial growth characteristics of the Houston-1 isolate were in contrast with those noted for the ATCC *Rochalimaea* type strains, which typically grew relatively rapidly without any delay in passaging, had shiny, smooth colonies, and were not similarly imbedded in the agar. Likewise, although *Rochalimaea* species isolates obtained from ATCC proliferated rapidly on the surface of cultured cells, initial Houston-1 isolate material did not produce a similar generalized infection when inoculated on Vero cell monolayers, thus suggesting that co-cultivation with eukaryotic cells is not the method of choice for primary isolation. The Houston-1 isolate, after additional laboratory passages on solid medium (and perhaps more analogous to the ATCC type strains in terms of more extensive passage history), was not retested for the ability to grow rapidly on eukaryotic monolayers.

After reinoculation of the organism onto either chocolate agar or TSA-sheep blood, there was no growth in air at 22°C or 42°C but good growth at 30°C and 35°C.

5 Colonies grew to slightly larger size when incubated in CO₂ (8%) at 35°C than when cultured without added CO₂ at 35°C. Growth on subculture was also achieved on HIA-rabbit blood or TSA-sheep blood when plates were incubated in candle jars as previously described for other *Rochalimaea* isolates by Slater et al. (30). There was no growth observed on Sabouraud-dextrose medium. The growth characteristics of the freshly isolated Houston-1 agent contrasted with those of well-established type species of *Rochalimaea*. With passaging, colony morphology and speed of growth of the novel agent began to more closely resemble those of other *Rochalimaea*-type species. Although *R. henselae* appears to be a fastidious and slow growing organism, it can be cultivated by standard laboratory procedures. Relatively rapid growth (4 days between subculture) of the Houston-1 isolate was achieved by multiple passaging of fresh colonies shortly after they initially became visible. Semi-automated, clinical bacterial isolation procedures, which often rely on liquid media-based assays, in the absence of exogenous gaseous carbon dioxide, may not be suitable for cultivation/detection of primary *Rochalimaea* isolates. Moreover, such cultures are generally not maintained for an incubation period sufficient to detect growth of a primary isolate.

30

Preliminary attempts to cultivate the Houston-1 isolate in stationary, liquid media did not produce turbid suspensions of individual organisms; however, the blood agar plate-derived inoculum material appeared to act as foci for growth of limited numbers of large cohesive

35

aggregates. Reinoculation of agar-grown organisms into Bactek 660 6A or 7A bottles (Becton Dickinson, Cockeysville, Md.) did not result in sufficient growth to change the growth index as compared to uninfected
5 controls.

2. Additional *Rochalimaea* isolates.

Four *Rochalimaea*-like isolates, previously submitted to
10 the Centers for Disease Control and Prevention (CDC) for microbial identification were compared with the Houston-1 isolate and recognized *Rochalimaea* species. Two of these isolates were recovered from patients in Oklahoma, one isolate originated in a patient who apparently acquired
15 his illness in Arkansas, and a fourth isolate which originated in San Diego County, California. This last isolate currently represents one of the first *Rochalimaea* isolates, that we are aware of, that has been made in recent years as well as one of the first isolates reported
20 from an HIV-infected individual (November, 1986).

C. Clinical Biochemical Analysis

25 Biochemical tests were performed by standard methods (16) and using the RapID ANA II System which tests for the presence of preformed enzymes (Innovative Diagnostic Systems, Inc., Atlanta, Ga.). Tests for motility included observation of growth characteristics in motility agar and
30 direct observation of bacilli with dark field microscopy. Presence of catalase was tested for by emulsifying a colony in hydrogen peroxide and checking for the presence of microscopic bubbles formed under a cover slip. The presence of oxidase was tested for using tetramethyl-p-
35 phenylenediamine.

Except for the production of peptidases, the Houston-1 isolate was biochemically inert when tested by typical clinical procedures. The RapID ANA II system, designed primarily for the clinical identification of anaerobic organisms by detection of specific preformed enzymes, is also useful for the identification of difficult to identify aerobic organisms. The RapID ANA II system, when used for analysis of the Houston-1 isolate, detected a limited number of enzyme-substrate cleavage reactions which included the cleavage of leucylglycine, glycine, proline, phenylalanine, arginine, and serine resulting in an identification number 000671. No known microbe is currently associated with this identification number, however, members of the genus *Rochalimaea* are not yet part of the commercial diagnostic database (*Rapid ID ANA II Code Compendium*, Innovative Diagnostic Systems, Atlanta, Georgia, 1989). Negative clinical assays included those testing for catalase, urease, esculin hydrolysis, motility, nitrate reduction, and oxidase.

20

D. Staining and Morphologic Characteristics

Four day-old cultures of the Houston-1 isolate were prepared for microscopy by flooding a blood agar plate containing the colonies with phosphate-buffered saline (PBS) and then gently sweeping adherent colonies off the agar surface with a bacteriological loop. A small aliquot of this material was placed directly on a clean microscope slide, heat-fixed, and stained with Gimenez stain. Other material was fixed with glutaraldehyde and prepared for electron microscopy. Briefly, the glutaraldehyde fixed material was filtered onto a Nucleopore filter (0.2 μ m pore size, Nucleopore Corp., Pleasanton, Calif.) and washed three times with Sorenson's buffer (pH 5.0). The filtered

35

material was treated in 1% osmium tetroxide for 2 hours and again washed three times with Sorenson's buffer. The specimens were dehydrated in a graded series of increasing concentrations of ethanol (30% to 100%). The dehydrated
5 specimens were immersed in hexamethyldisilazane (Polysciences, Inc., Warrington, Pa.) for 2 hours and then dried in a desiccator overnight. Finally, the specimens were placed on a stub, sputter coated with gold, and observed with a Philips (model 515) scanning electron
10 microscope.

Rapidly proliferating organisms from four day-old cultures, obtained after several subpassages, stained readily with Gimenez histological stain. Organisms so
15 stained appeared as small red bacilli, often slightly curved. Organisms obtained from older, but still quite viable colonies, resisted uptake of Gimenez stain. The material which was successfully used for light microscopy was also prepared for and observed using a scanning
20 electron microscope. As with the Gimenez-stained material, and the observations of growth habits noted during various culturing experiments, the organisms viewed with the scanning electron microscope appeared to form cohesive aggregates, with relatively few organisms
25 existing freely. The average size of organisms visualized was approximately 2 μ m in length by 0.5 to 0.6 μ m in width. All organisms observed within individual microscopic preparations, which presumably include the products of multiple generations, appeared to be relatively uniform in
30 size.

E. Fatty Acid Analysis

Whole cell fatty acid analysis was performed on *R. henselae*, sp. nov. (Houston-1) cultures incubated at 35°C in air and harvested after four days growth on chocolate agar. Fatty acid methyl esters were chromatographed on a Hewlett Packard series II 5890 gas chromatograph (Miller, L., T. Berger, "Bacterial identification by gas chromatography of whole cell fatty acids," Hewlett-Packard application note 228-41, Hewlett-Packard, Avondale, Pa., 1985) and identified using a computer-assisted comparison of retention times of the sample with that of a standard mixture (Microbial-ID, Newark, Del.).

The major fatty acids observed after whole cell fatty acid analysis of the Houston-1 isolate were octadecenoic acid (C_{18:1}, 54-56%), octadecanoic acid (C_{18:0}, 18-20%), and hexadecanoic acid (C_{16:0}, 17%). The absence of other detectable fatty acids excluded identification of almost all other bacteria except members of the genus *Brucella*. This fatty acid pattern was similar to that observed with *R. quintana* and other recent *Rochalimaea*-like isolates (30).

F. 16S rRNA Gene Sequence Analysis

1. DNA extraction, amplification and cloning.

DNA for polymerase chain reaction (PCR) amplification was extracted from pure cultures of *R. quintana*, *R. vinsonii*, and *R. henselae* (Houston-1 isolate) using sodium dodecyl sulfate (SDS)/proteinase K lysis followed by phenol/chloroform extraction as previously described (29). The resulting aqueous phase was concentrated using a Centricon 30 concentrator (Amicon Corp., Danvers, Mass.) and washed three times with 2ml of TES (10mM Tris, pH 8.0; 1mM EDTA; 10mM NaCl).

PCR amplification was performed using a thermal cycler and GeneAmp reagents (Perkin Elmer-Cetus, Norwalk, Conn.). Two pairs of "universal," degenerate primers known to amplify approximately 92% of the 16S ribosomal RNA gene, as two separate PCR products, from all eubacteria previously studied were used to prime PCR synthesis of products that were subsequently used for cloning and sequence analysis. The 5' end of each primer was modified to contain unique restriction endonuclease sites to facilitate cloning. Each sample was amplified for three cycles at: 94°C, 1 minute; 48°C, 2 minutes; 66°C, 1 minute 30 seconds, followed by 27 cycles at: 88°C, 1 minute; 52°C, 2 minutes; 68°C, 1 minute 30 seconds.

The resulting PCR products were isolated from a 1.0% agarose gel and cloned into pUC 19 (29). Clones were sequenced using double-stranded sequencing with T7 DNA polymerase (SEQUENASE, U.S. Biochemicals, Cleveland, Ohio). Each isolate was amplified, cloned, and sequenced at least twice to prevent the reading of PCR incorporation errors; if discrepancies were detected, a third, independent sequence was produced. Great care was taken not to introduce contaminating bacterial DNA into the PCR reactions using the universal primers because of their broad range of amplification. GenBank accession numbers for the respective 16S rRNA gene sequences are as follows: *R. quintana*, M73228; *R. vinsonii*, M73230; *R. henselae* (submitted as *R. americana*), M73229.

Universal primers allowed amplification of approximately 1400 nucleotides of the rRNA gene sequence as two separate PCR products. 767-base pair (bp) products, corresponding to the 5' half of the 16S rRNA gene, produced using primers EC11 and EC12 (modified versions of P0mod and PC3mod primers used by Wilson et al.

(26) were observed when the Houston-1 isolate, *R. quintana* and *R. vinsonii* were amplified. No product was observed when these primers were used to amplify a negative control containing no DNA template. Similarly, a 737 bp product
5 corresponding to the 3' half of the 16S rRNA gene, produced with primers EC9 and EC10 (modified versions primers P3mod and PC5 used by Wilson et al. (40) was seen when using Houston-1 isolate, *R. quintana*, *R. vinsonii*. No PCR product was seen in the no DNA control. These PCR
10 products were cloned and sequenced.

2. DNA sequencing.

The 16S rRNA gene sequences used for comparison and
15 alignment were obtained by taking a consensus of three independent sequences for each cloned PCR product. The first and second sequences obtained for the Houston-1 isolate had three nucleotides in disagreement, and the first and second sequences for *R. vinsonii* had two
20 ambiguities. In both cases a third sequence agreed with one of the two previous sequences at these ambiguous positions and was taken as the consensus. The occasional disagreement among sequences was assumed to be the result of polymerase-nucleotide incorporation errors. The entire
25 sequence was used for alignment using the Gap program of the Genetics Computer Group. The sequence of the Houston-1 isolate was compared with 16S rRNA gene sequences on file with GenBank and showed the greatest homology with *R. quintana* (98.7%) and lesser homologies with 16S rRNA gene
30 sequences from organisms more distantly related (Table 1).

In our laboratory, we sequenced the 16S rRNA gene from *R. quintana* (Fuller strain) and found it to differ slightly from the sequence previously reported by Weisberg
35 et al. (35) and obtained from GenBank. Using our data, we

found the 16S rRNA gene sequence from the Houston-1 isolate to be 98.7% related to *R. quintana* and 99.3% related to the *R. vinsonii*. The *R. quintana* and *R. vinsonii* sequences were found to be 98.9% related. The
5 0.7% 16S rRNA gene sequence divergence seen between the Houston-1 isolate and *R. vinsonii* is greater than the 0.5% divergence reported for *Rickettsia prowazekii* and *Rickettsia typhi*. These two species of *Rickettsia* are clearly distinct species among the order *Rickettsiales*, to
10 which *Rochalimaea* belong.

The partial 16S rRNA gene sequence determined by Relman et al. (28) (GenBank Acc. #M59459) for the putative etiologic agent of BA was found to be identical to the
15 corresponding portion of the 16S rRNA gene sequence obtained from the Houston-1 isolate of *R. henselae*, sp. nov. (Table 1). Partial 16S rRNA gene sequences obtained from one of the Oklahoma isolates are identical to 16S rRNA gene sequences obtained from the Houston-1 isolate.
20 These completely homologous sequences indicate that the causative agents are one and the same species. The variation between 16S rRNA gene sequences noted between the Houston-1 isolate and other type species of *Rochalimaea* (Table 1) indicates that the Houston-1 isolate
25 represents a new species within the genus *Rochalimaea*.

Additionally, the *R. henselae* 16S rRNA gene sequence is present in CSD skin test antigens that have been used for diagnosis of this disease for many years.

30

Thus, the nucleic acid encoding the 16S rRNA subunit is specific for *R. henselae* and can be compared against the 16S rRNA DNA sequences of other organisms or in test samples to detect the presence of *R. henselae*.

Table 1. Relatedness between the Houston-1 isolate 16S rRNA gene and various eubacteria

Species ^a	% Homology with Houston-1 Isolate <i>Rochalimaea henselae</i>
BA-TF ^b	100.0
<i>Rochalimaea vinsonii</i>	99.3
<i>Rochalimaea quintana</i>	98.7
<i>Bartonella bacilliformis</i>	98.2
<i>Brucella abortus</i>	94.0
Cat scratch fever agent (AFIP)	87.9
<i>Rickettsia rickettsii</i>	84.9
<i>Ehrlichia risticii</i>	84.9

^a The entire 16S rRNA gene sequence (when available) was used for alignment. The *R. henselae*, Houston-1 isolate, *R. vinsonii*, and *R. quintana* sequences were determined in our laboratory, all other sequences were obtained from GenBank.

^b Partial 16S rRNA gene sequence from Relman et al. (28).

G. Citrate Synthase Gene PCR/RFLP Analysis

Restriction-endonuclease length polymorphism (RFLP) analysis was applied to PCR-amplified DNA, which was
5 primed with nondegenerate oligonucleotides previously demonstrated to initiate synthesis of PCR products approximately 381 nucleotides long from a portion of the rickettsial citrate synthase gene (25). Chromosomal DNA from *Rickettsia prowazekii* was used as a positive control
10 for PCR synthesis and digestion; controls containing no DNA template were always included in PCR amplifications.

1. DNA digestion and electrophoresis.

15 RFLP analysis of specific genes, amplified by the PCR technique, is useful for identifying rickettsial genotypes and species. Oligonucleotides, previously demonstrated to be suitable for priming PCR amplification of a portion of the citrate synthase genes from nearly all rickettsial
20 species, as well as from *R. quintana*, were tested for their ability to prime DNA amplification from DNA purified from the Houston-1 isolate and *R. vinsonii*. PCR products were readily produced using conditions comparable to those previously reported. Briefly, PCR amplification was
25 accomplished in 100- μ l volumes, using the protocols supplied with the GeneAmp DNA amplification reagent kit (Perkin-Elmer Cetus, Norwalk, Connecticut). Typically, 1 μ l of undiluted cytoplasmic extract DNA was used as PCR template. DNA amplification was done in a Perkin-Elmer
30 Cetus DNA Thermal Cycler, using 35 cycles of denaturation (20 seconds at 95°C), annealing (30 seconds at 48°C), and extension (2 minutes at 60°C).

PCR amplification of DNA was verified by rapid agarose
35 electrophoresis of a small amount of PCR product.

Restriction and endonuclease digestion was done with 20 μ l of PCR reaction mixture, following standard techniques (29) and incubations were at 37°C. All restriction endonucleases were obtained from New England BioLabs, Beverly, Massachusetts. After addition of dye-Ficoll loading mixture (29), the digested reactions were loaded on 1.5mm thick, 8% polyacrylamide vertical gels (Bio-Rad Laboratories, Richmond, California) made by standard procedures (29). Gels were run at 80 volts for 4 hours in simple vertical electrophoresis chambers (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, Maryland). The gels were then stained with ethidium bromide prior to illumination on a UV light source (365nm; Spectronic Corp., Westbury, New York) and photographed with Polaroid type 655 P/N film (Polaroid Corp., Cambridge, Massachusetts).

Digested DNA fragments were separated and analyzed using standard electrophoretic protocols and methods previously described by Regnery et al. (25). The number of comigrating DNA fragments, observed between homologous PCR/RFLP digests of two or more isolates, were counted. Data from the number of comigrating DNA fragments were used to derive estimates of sequence relatedness by methods described by Upholt (32) and subsequently used by others to estimate sequence divergence between related bacteria.

All three of the uncut *Rochalimaea* citrate synthase PCR products were slightly larger (approximately 400bp) than those produced for members of the genus *Rickettsia* (approximately 381bp). Variation was noted between the sizes of PCR-amplified citrate synthase products obtained from different *Rochalimaea* isolates. PCR-amplified products were digested with seven restriction

endonucleases and subjected to polyacrylamide gel electrophoresis. Obvious differences were seen in many of the digest patterns of PCR-amplified citrate synthase sequences from the various isolates; PCR/RFLP analysis
5 allowed for rapid differentiation of other isolate genotypes.

The numbers of DNA fragments produced by digestion of the PCR-amplified, citrate synthase-specific DNA with
10 seven restriction endonucleases are tabulated in Fig. 1, together with the number of comigrating fragments. Estimates of sequence divergence derived by numerical analysis of the percentage of comigrating fragments illustrate that all of the isolates examined have
15 substantial inferred citrate synthase sequence divergence (6 to 11%) equalling or exceeding similar estimates for citrate synthase sequence divergence among recognized rickettsial species (e.g., 2 to 6%).

20 PCR/RFLP analysis clearly differentiated *R. henselae*, sp. nov., genotype from that of either *R. quintana* or *R. vinsonii*. Multiple restriction-endonuclease digests of the citrate synthase-specific PCR products from other *Rochalimaea*-like isolates from Oklahoma (two isolates),
25 Arkansas (one isolate), and Southern California (one isolate) demonstrated that all of the isolates studied are identical to one another, and *R. henselae* (Houston-1 isolate), according to the PCR/RFLP methods applied herein.

30 It is clear that in addition to cat scratch disease and bacillary angiomatosis the disease spectrum of this organism may be variable and include a syndrome of fever and bacteremia and bacillary peliosis hepatis. Thus, the
35 nucleic acid methods described herein can be used to

detect the presence of *R. henselae* associated with these disease syndromes.

EXAMPLE 2

5

Serological Methods

10 An immunofluorescent assay (IFA) test was developed to detect antibodies specifically reactive with *R. henselae* antigen in order to begin to assess distribution and prevalence of infection, and also to help define the full spectrum of *R. henselae*-induced disease. Infectious
15 organisms were rendered nonpathogenic by inactivation by gamma irradiation.

A. Preparation of *R. henselae* antigenic determinant

20 *R. henselae* bacilli cultivated on erythrocyte-enriched agar media, and then kept in solution, tend to auto-agglutinate as previously described; this clumping obstructs the production of a well dispersed IFA antigen. Inhibition of auto-agglutination was achieved by co-
25 cultivation of *R. henselae* with Vero cells to which individual *Rochalimaea* organisms avidly adhered. Briefly, *R. henselae* cells are cultured in liquid medium with Vero cells for 4 days. After decanting most of the liquid medium, glass beads are added to the culture flask and
30 gently agitated in the remaining medium. This agitation with beads loosens the Vero cells and their adherent *R. henselae* cells from the flask walls. The *R. henselae* cells complexed with the Vero cells are then inactivated (rendered nonpathogenic) by gamma irradiation. Antigen
35 and antisera were prepared for IFA testing by standard techniques.

B. Preparation of antisera (antibodies)

Briefly, the *R. henselae* antigen obtained from isolated *R. henselae* cultures and suspended in PBS is inoculated
5 into a rabbit to cause the rabbit to produce antibodies specifically reactive with the antigen. A blood sample from the animal is taken and red blood cells are removed to obtain antisera. The serum containing *R. henselae* antibodies is then subjected to ammonium sulfate to
10 precipitate gamma globulins (IgG) out of the antiserum.

C. IFA

The IFA of this example is conducted briefly as
15 follows: The Vero cell-associated *R. henselae* antigenic determinant prepared above is spotted into a well of a 12-well microscope slide and a second spot of *R. quintana* (Fuller isolate) is also placed in the well. The spots are air dried and then acetone fixed for 10 minutes.
20 Serial dilutions of the antisera being tested (eg. 1/32, 1/64, etc., dilution endpoint) are placed in the paired wells with the antigen. The slides are then incubated in a moist chamber at 37°C for 30 minutes and thereafter washed 3 times with PBS, rinsed with distilled water and
25 air dried. Fluorescein labeled goat antihuman IgG is then spotted into each well, and the slides incubated, washed, rinsed and dried as above. Buffered glycerol is added to the wells for optical enhancement and the slides are then analyzed by fluorescence microscopy to detect the presence
30 of antibody specifically reactive with *R. henselae* antigen.

In an alternative method, the *R. henselae* specific antibody purified above can be directly labeled with a
35 detectable moiety such as fluorescein (14).

In all IFA determinations, antisera from humans with culture-confirmed *R. henselae* or *R. quintana* infections were used as positive controls.

5 Sera from 40 patients with suspected cat scratch
disease were evaluated by IFA for reactivity with *R.*
henselae antigen. Thirty-five (87.5%) patients had
antibody titers to *R. henselae* that were equal to, or
exceeded, 1/64 serum end-point dilution (Fig. 2). Many
10 patients had sera with titers exceeding 1/1024. Sera
collected during acute and convalescent phases of illness
were available from several patients. Of five sets of
paired sera that had different titers and included at
least one specimen with a titer equal to, or exceeding,
15 1/64, three demonstrated four-fold rises or falls in
antibody titer. Three additional paired sets of sera
could not be evaluated for change in titer because both
sera had antibody specifically reactive with *R. henselae*
antigen of, or exceeding, a titer of 1/1024 (the maximum
20 titer assayed). Eight of the sera with a titer of, or
exceeding, 1/64 also had low antibody titers to *R.*
quintana which did not exceed 1/32. In each of these
sera, the titer of antibody specifically reactive with *R.*
henselae exceeded the titer to *R. quintana* by at least
25 four-fold.

107 sera collected from persons who identified
themselves as healthy individuals were obtained from a
contract vendor (Worldwide Biologics, Cincinnati, OH).
30 When these sera were tested by IFA for antibody reactive
with *R. henselae* and *R. quintana*, 101 (94%) had titers
less than 1/64 (Fig. 3). Of the six sera that had
antibody titers to *R. henselae* antigen equal to or greater
than 1/64, three had considerably elevated antibody titers

(i.e., 1/512 and 1/1024). Antibody titers to *R. quintana* exceeding 1/16 were not detected among the serum donors.

Sera from persons with a variety of diseases were
5 evaluated for the presence of possible antibodies
specifically reactive with *R. henselae*. Titers less than
or equal to 1/64 were detected in two of ten persons with
brucellosis, however, the two low level positive serologic
responses did not correlate with increasing titers of
10 antibody to *Brucella abortus* as detected by
microagglutination. One of three sera from patients with
Lyme disease had a titer of 1/64 to *R. henselae*. Sera
from patients with tularemia and sera from patients with
Yersinia enterocolitica infections did not show antibody
15 titers to *R. henselae* that were in equal to or greater
than 1/64. A number of other reference human antibodies
used as reagents in diagnostic kits were evaluated with
the *R. henselae* IFA test. None of these sera showed a
titer of antibody for *R. henselae* at or above 1/64. The
20 reference sera included human antisera to: *Mycoplasma*
pneumoniae, *Treponema pallidum*, *Coxiella burnetii*,
Ehrlichia chaffeensis, chlamydia group, spotted fever
group rickettsiae, typhus group rickettsiae, varicella
zoster, influenza type A, adenovirus, dengue virus type 2,
25 herpes simplex, coxsackievirus group A, poliovirus type 2,
cytomegalovirus, rubella, human immunodeficiency virus
type I, as well as alpha-fetoprotein and rheumatoid
factors.

30 Sera containing high-titered human antibody
specifically reactive with *R. henselae* and antibodies for
R. quintana did not react with "*A. felis*" antigen in the
IFA test. Hyperimmune rabbit antisera and monoclonal

antibodies directed against "*A. felis*" antigen were not reactive with *R. henselae* whole cell antigen.

High titered *R. quintana* antibody (1/1024 dilution
5 endpoint) obtained from a human volunteer infected with *R. quintana* (Fuller isolate) yielded no discernable reaction with *R. henselae* antigen (<1/16 dilution endpoint). Similarly, minimal (<1/32 dilution endpoint) *R. quintana* antibody titers were noted when high titered (e.g. >1/1024
10 dilution endpoint) serum was used from a culture positive *R. henselae*-infected patient.

Thus, it is seen that the human serologic responses to *R. henselae* and *R. quintana* (Fuller isolate) antigens, as
15 assayed in the IFA test, are species-specific and it is unlikely that the antibody reactions observed with *R. henselae* antigen were due to antigenic stimulation by any species other than *R. henselae*.

20 There was a low prevalence of significantly elevated levels of antibody specifically reactive with *R. henselae* found among apparently healthy serum donors, indicating that *R. henselae* infection may be relatively common.

25 Out of 40 patients clinically diagnosed with cat scratch disease, 35 (87.5%) had sera antibody titers to *R. henselae* antigen that equaled or exceeded 1/64 and several paired sets of sera showed four-fold changes in titer. This method of detecting *R. henselae* antigen or antibodies
30 specifically reactive therewith provides a useful diagnostic tool for identification of patients with cat scratch disease and thereby reduces reliance on clinical diagnosis alone, use of non-pharmaceutically approved CSD

skin test antigen preparations, and need for surgical biopsy.

The method of diagnosing cat scratch disease exemplified herein can be applied equally effectively to the diagnosis of bacillary angiomatosis, because an etiologic agent of both diseases is *R. henselae*. Also, because *R. henselae* infection is associated with other disease syndromes, such as a syndrome of fever and bacteremia and bacillary peliosis hepatis, the serological, immunocytochemical, cytological and nucleic acid detection methods described above can be effectively used to diagnose these diseases.

15

EXAMPLE 3

Detection of *R. henselae* and *R. quintana* by PCR

20 A. Bacterial Strains

All strains of bacteria used for evaluating the specificity of the PCR and hybridization assay are listed in Table 2. *Rochalimaea* spp. were grown on heart infusion agar plates supplemented with 5% defibrinated rabbit blood (HIA-RB) (BBL, Rockville, Md) incubated for 3 to 5 days at 34°C in the presence of 5% CO₂. *Bartonella bacilliformis* was cultivated on HIA-RB for 6 to 8 days at 28°C without supplemental CO₂. *A. felis* was grown on charcoal-yeast extract agar plates (Carr-Scarborough Microbiologicals, Decatur, Ga.) for 2 to 3 days at 32°C without CO₂.

Table 2. Isolates whose DNA was used for specificity testing of the PCR primers and oligonucleotide probes.

ID	bacteria	source (ref.)	Probes		
			PCR	RH1	RQ1
Houston-1*	<i>R. henselae</i>	HIV+ patient (23)	+	+	-
San Ant-1	<i>R. henselae</i>	HIV- patient (18)	+	+	-
San Ant-2	<i>R. henselae</i>	CSD patient (9)	+	+	-
San Ant-3	<i>R. henselae</i>	CSD patient (9)	+	+	-
San Diego-2	<i>R. henselae</i>	San Diego, HIV+	+	+	-
OK88-64	<i>R. henselae</i>	HIV+ patient (34)	+	+	-
OK88-712	<i>R. henselae</i>	HIV- patient (34)	+	+	-
OK89-674	<i>R. henselae</i>	HIV- patient (34)	+	+	-
OK89-675	<i>R. henselae</i>	HIV- patient (34)	+	+	-
OK90-615	<i>R. henselae</i>	HIV- patient (34)	+	+	-
OK90-782	<i>R. henselae</i>	HIV+ patient (34)	+	+	-
CAL-1	<i>R. henselae</i>	San Diego, HIV+	+	+	-
Fuller *	<i>R. quintana</i>	ATCC VR358	+	-	+
OK90-268	<i>R. quintana</i>	HIV+ (34)	+	-	+
SH-PERM	<i>R. quintana</i>	Russia	+	-	+
D-PERM	<i>R. quintana</i>	Russia	+	-	+
F9251 *	<i>R. elizabethae</i>	heart valve (8)	- ^a	-	-
RV *	<i>R. vinsonii</i>	ATCC VR152	-	-	-
KC584	<i>B. bacilliformis</i>	ATCC 35686	-	-	-
BV *	<i>A. felis</i>	ATCC 53690 (7)	-	-	-

^a The 414bp PCR product was not observed. A larger product of approximately 1300 bp was amplified from *R. elizabethae* that failed to hybridize with either the RH1 or RQ1 probe.

* Denotes type strain for the species.

B. Clinical Samples

Twenty-five samples from patients clinically diagnosed with CSD were used for evaluating a further example of a PCR assay (Table 3). All CSD cases were clinically diagnosed by the physician and had regional lymphadenopathy and cat contact in the absence of other obvious diagnosis. All patients whose samples were used met this definition except patient #16 (Table 3), who had no known history of cat contact. Sixteen of these were fresh lymph node biopsy specimens and nine were lymph node aspirates. In addition, five lymph node aspirates from non-CSD patients, from whom other organisms were isolated, were included as negative controls. Likewise, three lymph node biopsies from non-CSD patients were used as additional negative controls. Serology was performed on some patients (when serum was available) by the indirect fluorescence antibody test illustrated in Example 2.

Fresh frozen tissue from both lymph node biopsy specimens and lymph node aspirates were suitable samples.

Table 3. Information and PCR/dot-blot results on CSD patients.

patient	state	sample	PCR-CSD	hybridization with probe:		serology ^a
				RH1	RQ1	
1	MA	aspirate	+	+	-	+
2	MA	biopsy	+	+	-	+
3	MO	biopsy	+	+	-	+
4	FL	biopsy	+	+	-	+
5	FL	biopsy	+	+	-	+
6	OH	biopsy	+	+	-	+
7	SC	biopsy	-	-	-	+
8	NJ	biopsy	+	+	-	+
9	VA	biopsy	-	-	-	+
10	NJ	biopsy	+	+	-	+
11	NJ	biopsy	+	+	-	+
12	PA	biopsy	+	+	-	+
13	MA	aspirate	+	+	-	+
14	WV	biopsy	-	-	-	+
15	ME	biopsy	+	+	-	+
16	NC	biopsy	+	+	-	+
17	WA	biopsy	+	+	-	+
18	MA	aspirate	+	+	-	+
19	GA	biopsy	-	-	-	-
20	TN	aspirate	+	+	-	+
21	TN	aspirate	+	+	-	+
22	TN	aspirate	+	+	-	+
23	FL	aspirate	+	+	-	ND
24	TN	aspirate	+	+	-	ND
25	VA	aspirate	+	+	-	ND
26	TN	aspirate	- ^b	-	-	ND
27	TN	aspirate	- ^b	-	-	ND

^a serology was performed by the indirect fluorescence antibody test as previously described (23). An anti-*Rochalimaea* titer of 1:64 or higher was considered positive.

^b Two representative negative controls of non-CSD cases from which other bacteria were isolated.

C. DNA Extraction

DNA was extracted from bacterial cells, lymph node tissue, or lymph node aspirates using modifications of a procedure previously described (4). Briefly, bacterial growth harvested from approximately 1/8th of a standard size (85mm) HIA-RB plate was resuspended in 300µl of PCR diluent (10mM Tris, 10mM NaCl, 1mM EDTA, pH 8.0). For lymph node tissue, samples (approximately 100mg) were dispersed using a disposable homogenizer in minimal essential medium, (0.5ml) and 50 to 100µl of this homogenate was diluted to 300µl with PCR diluent. Lymph node aspirates (50 to 100µl) were resuspended and diluted to 300µl with PCR diluent. The samples were then made 1.0% sodium dodecyl sulfate (SDS) and proteinase K was added to a final concentration of 100ng/µl, and the samples were incubated for 2 hours at 55°C. After incubation, the lysates were extracted three to four times with a 50:50 mixture of buffer saturated phenol and chloroform/isoamyl alcohol (24:1). The resulting aqueous supernatant was diluted to 2.0ml with PCR diluent, filtered through a Centricon 30 filter (Amicon, Danver Mass.) and washed twice more with 2.0ml aliquots of PCR diluent. The subsequent filter retentate (average volume of 40µl) was collected and used as template for the PCR. For every DNA extraction run, a reagent blank was processed exactly as described above to ensure that all extraction buffers and reagents were not contaminated with *Rochalimaea* DNA.

D. PCR Primer and Hybridization Probe Design

A library of *R. henselae* DNA was constructed in the vector lambda ZapII (5). The library was screened with either a pool of eight monoclonal antibodies or rabbit hyperimmune serum for expression of antigenic proteins. A clone expressing a 60-kilodalton antigen reactive with rabbit

anti-*R. henselae* serum has been isolated and the gene sequenced (6) (GenBank Accession L20127). The deduced amino acid sequence was shown to have 37% sequence homology (over the entire sequence) with the *htrA* locus described from

5 *Escherichia coli* (17). Primer pair CAT1 5' GATTCAATTGGTTTGAA(G and A)GAGGCT 3' (SEQ ID NOs:1 and 2) and CAT2 5' TCACATCACCAGG(A and G)CGTATTC 3' (SEQ ID NOs:3 and 4) (Fig. 4), which defines a 414 base pair (bp) fragment from both *R. henselae* and *R. quintana*, was used for PCR

10 amplification. Twenty-base pair oligonucleotide probes RH1 (SEQ ID NO:5) and RQ1 (SEQ ID NO:6) (Fig. 4) were used as species-specific hybridization probes.

Partial nucleotide sequences (150-200 nucleotides) of

15 the same gene from the other three species of *Rochalimaea* were obtained using conserved PCR primers. PCR with the primer pair *htr5* (5' AATCTAGATTGCTTTTCGCTATTCCGGC 3' (SEQ ID NO:8)) and *htr6* (5' AAGGATCCATTTGTTTCGCACTTGTAGAAG 3' (SEQ ID NO:9)) resulted in the amplification of a 650 base pair

20 product from each of the four *Rochalimaea* species. The 150-200 base pair sequences of the other three species obtained from these amplification products were found to be 85 to 92% conserved with the *R. henselae* sequence. No evidence of the present *htrA* gene was found in *B. bacilliformis*, an organism

25 phylogenetically closely related to *Rochalimaea* spp. (21, 27). This observation is interesting since *B. bacilliformis* does not grow at elevated temperatures, a trait which in part may be due to the lack of a functional *htrA* gene product.

30 E. PCR Assays

DNA prepared from bacteria, fresh lymph node tissue, or lymph node aspirates was used as template for the PCR assays. Five µl portions of the template DNA (undiluted and diluted

1:10) extracted from the clinical samples was used for each PCR assay. The approximate concentration of DNA extracted from bacterial isolates was determined by agarose gel electrophoresis next to known quantities of standard DNA.

5 Diluted bacterial DNA (approximately 1ng) was used for the initial determination of primer specificity. For subsequent PCR on clinical samples, 10pg (in 10µl) of DNA extracted from either *R. henselae* or *R. quintana* was used as a positive control and the DNA extraction blank and water (10µl each)

10 were used as a negative controls. The GeneAmp reagent (Perkin-Elmer Cetus, Norwalk, Conn.) kit was used for all PCR assays. Degenerate primer pair CAT1 and CAT2 was used to prime the polymerization reactions. The primer mixture included about equal amounts of the *R. quintana* (SEQ ID NOs:2

15 and 4) and *R. henselae* (SEQ ID NOs. 1 and 3) primer sequences. Amplification was accomplished by predenaturing for 5 minutes at 94°C followed by 35 cycles of 94°C, 30 seconds; 50°C for 60 seconds; and 70°C for 45 seconds in a model 9600 thermal cycler (Perkin-Elmer). Ten microliters

20 from each PCR assay was electrophoresed through a 1.2% agarose gel, stained with ethidium bromide, and photographed. The presence of a 414bp band was considered positive. Each sample of DNA extracted from the clinical specimens was also amplified with primer pair GHPCR1 and GHPCR2 (36). This

25 primer pair amplifies a 422bp fragment from a conserved region of the human growth hormone gene and serves as a positive control for successful extraction of amplifiable DNA. DNA extracts from clinical samples that failed to amplify with primer pair GHPCR1 and GHPCR2 were excluded from

30 further study.

F. Specificity of the PCR Assay

Under the conditions described above and with purified

35 template DNA, all 12 *R. henselae* isolates and all four *R.*

quintana isolates yielded the predicted 414bp fragment of amplified DNA (Table 2). No amplification products were observed for *R. vinsonii*, *B. bacilliformis*, and *A. felis*. *R. elizabethae* was amplified with this primer pair, but the product was much larger (approximately 1300bp) than the 414bp predicted for *R. henselae* and *R. quintana*. Thus, the 414bp PCR product appears to be specific for *R. henselae* and *R. quintana*. A PCR product of approximately 50 to 60bp was occasionally observed in the no DNA control and presumably corresponds to primer dimer.

The degenerate primers CAT1 and CAT2 appear to be well conserved within the isolates of *R. henselae* and *R. quintana*

Greater success was obtained using aspirates (9/9, 100% positive) than biopsies (12/16, 75% positive), probably because of the inherent difference between nodes which are fluctuant and thus can be aspirated and those which are not. Difficulty is encountered in standardizing the amount of DNA extracted from either lymph nodes or aspirates. Since we utilized a total lysate procedure, both RNA and DNA was obtained and measuring the lysates absorbance would be a poor indicator of DNA concentration. Accordingly, we used each sample of template undiluted and at a 1:10 dilution for amplification.

G. Dot-blot Hybridizations

To confirm the identity of the PCR products and to allow differentiation of products amplified from *R. henselae* and *R. quintana* templates, a dot-blot hybridization assay was performed on the PCR products amplified from the bacteria listed in Table 2. Oligonucleotide probes RH1 and RQ1 were used for this purpose. RH1 and RQ1 were nonisotopically

labeled by transfer of a digoxigenin-ddUTP nucleotide to the 3' end of each oligonucleotide by means of terminal transferase using Genius 5 labeling kit (Boehringer Mannheim, Indianapolis, Ind.). For the dot-blot hybridization assays, 5 5µl of each PCR product was denatured for 10 minutes by the addition of 0.5µl of 4M NaOH containing 100mM ethylene diamine tetraacetic acid. One-microliter aliquots were spotted onto each of two nylon membranes (Boehringer Mannheim) and the DNA was cross-linked to the nylon by UV 10 irradiation (Stratalinker, Stratagene, La Jolla, Calif.). The nylon membranes were then blocked for 1 hour at 62°C, using standard prehybridization solution from the Genius 7, luminescent detection kit (Boehringer Mannheim). Standard hybridization solution was 5X SSC (1X SSC= 0.15M NaCl and 15 0.015M sodium citrate) containing 0.1% N-laurylsarcosine, 0.02% SDS, and 1.0% blocking reagent (Boehringer Mannheim). Hybridization was then performed at 62°C ($T_m - 8^\circ\text{C}$ for both probes) for 1 hour in fresh prehybridization solution containing either probe RH1 or RQ1 at a concentration of 2 20 pmol/ml. The hybridized membrane was then washed twice for 15 minutes each in 2X SSC containing 0.1% SDS at room temperature, followed by two washes of 15 minutes each at 52°C in 0.5X SSC with 0.1% SDS. The hybridized filter was then blocked, reacted with alkaline phosphatase conjugated 25 antibody, washed, and soaked in Lumigen PPD chemiluminescent substrate according to the manufacturer's directions (Genius 7 kit, Boehringer Mannheim). The resulting filter was exposed to X-ray film for 5 to 20 minutes and the film was developed.

30

H. Specificity of Dot-Blot Hybridization Assay

PCR products amplified from all 12 isolates of *R. henselae* hybridized with probe RH1. PCR products from all 35 four isolates of *R. quintana* hybridized only with probe RQ1.

Neither probes RH1 or RQ1 hybridized to the PCR products from *R. elizabethae*, *R. vinsonii*, *B. bacilliformis*, or *A. felis*. Thus, the dot-blot hybridization assay allows differentiation between PCR products amplified from *R. henselae* and *R.*

5 *quintana*.

The oligonucleotide probes (RH1 and RQ1) while being species-specific, appear to be well-conserved within the species.

10

I. PCR and Dot-Blot Assays on Clinical Samples

To evaluate the PCR and dot blot assays for detection of *R. henselae* and *R. quintana* in clinical samples, these
15 techniques were applied to 16 samples of fresh lymph node tissue and 9 aspirates from CSD cases. Twenty-one of 25 samples (84%) produced the 414-bp product that is characteristic of *R. henselae* or *R. quintana* (Table 3). Representative PCR products obtained from lymph node biopsy
20 samples and a lymph node aspirates of suspect CSD patients were electrophoresed. Two samples produced the characteristic 414bp band only when the template DNA was diluted 1:10 before amplification. Typical of these samples is #9, the amplification of which appears to be inhibited by
25 large amounts of leukocyte DNA. When the sample containing the template DNA was diluted 1:10 prior to amplification, the 414bp band was clearly produced. The characteristic 414bp fragment was not amplified from any of the eight lymph node tissue samples from non-CSD cases or from DNA extraction
30 blanks.

To confirm the identity of the PCR products amplified from the clinical samples and to sort those infections caused by *R. henselae* from those caused by *R. quintana*, a dot-blot

hybridization was performed using species-specific probes RH1 and RQ1. The PCR products from all 21 samples that amplified to produce the characteristic 414bp fragment hybridized with *R. henselae*-specific probe RH1. Conversely, none of these
5 samples hybridized with *R. quintana*-specific probe RQ1. Thus, all the PCR positive samples studied here, from suspected CSD patients in 11 different states (Table 3), appear to be associated with *R. henselae* and not *R. quintana*. None of the samples that failed to amplify the 414bp fragment
10 as determined by agarose gel electrophoresis hybridized with either probe.

The present results indicate that, unlike BA and other opportunistic infections seen among AIDS patients that may be
15 caused in some cases by *R. henselae* and in others by *R. quintana*, CSD appears to be caused primarily (or perhaps exclusively) by *R. henselae*.

The 84% positive samples from suspect CSD cases for the
20 PCR assay described here is virtually identical to the 84% and 88% positive observed by serologic means on two separate groups of samples from suspect CSD patients (24, 37). Twenty-two of the samples from CSD cases tested herein by PCR were also tested by serology (Table 3). Twenty-one of these
25 (95%) had an IFA titer of 1:64 or greater. Thus, three samples collected from seropositive individuals were negative by the PCR assay. This apparent discrepancy may be due in part to the lack of intact organisms in the lymph nodes from patients in the later stages of CSD. In fact Gerber et al
30 have postulated that the lingering cell-mediated immune response and resulting granulomatous reaction rather than bacterial invasion may be the major pathogenic mechanism of CSD (13). Alternatively, there may be inhibitors of PCR

present in lymph node tissue that preclude attaining optimal sensitivity of the assay.

The PCR assay offers the advantage of early diagnosis
5 since it is not dependent on the patient mounting a
detectable humoral immune response. In addition, the PCR
assay differentiates *R. henselae* from *R. quintana* infections.
A rapid and specific test for CSD affords the clinician an
alternative to culture or serology for laboratory
10 confirmation of the diagnosis, thereby permitting the
clinician to rule out malignancies such as lymphoma and to
consider antibiotic therapy. Although the efficacy of
antibiotics in treating CSD remains uncertain, successful
treatment with four antibiotics (rifampin, ciprofloxacin,
15 trimethoprim-sulfamethoxazole, and gentamicin sulfate) has
been reported (19), and in vitro, *R. henselae* is sensitive to
many common antibiotics (9).

20

EXAMPLE 4

Identification of Antigenic Fragments of *R. henselae*

A library of *Rochalimaea henselae* DNA was constructed
25 in the cloning vector lambda ZapII and screened for
expression of antigenic proteins using a pool of sera from
patients diagnosed with cat-scratch disease (CSD) and who had
antibodies to *Rochalimaea* as determined by the indirect
fluorescent antibody (IFA) assay. Ten immunoreactive phage
30 were subcloned as recombinant plasmids by in vivo excision.
All ten recombinants expressed a protein of approximately
17-kilodaltons (kDa) when examined by immunoblot analysis
using the pool of human sera. Restriction endonuclease
digestion of each of the ten recombinant plasmids indicated

seven different profiles, suggesting that cloning bias was not the reason for repeatedly isolating clones expressing the 17-kDa antigen. The gene coding for the 17-kDa antigen was sequenced and shown to code for an open reading frame of 148 amino acids and a predicted molecular mass of 16,893 Daltons. The amino terminus of the deduced amino acid sequence was hydrophobic in nature and similar in size and composition to signal peptides found in Gram-negative bacteria. The remainder of the deduced amino acid sequence was more hydrophilic and may represent surface-exposed epitopes. Further subcloning of the 17-kDa antigen as a biotinylated fusion protein in the expression vector PinPoint Xa-2 resulted in a 30-kDa protein that was highly reactive on immunoblots with individual serum samples from patients with CSD. The agreement between reactivity with the 30-kDa fusion protein on immunoblot analysis and results obtained by the IFA assay was 92%, n=13 for IFA positive and 88%, n=9 for IFA negative. The recombinant expressed 17-kDa protein should be of value as an antigen for serologic diagnosis of CSD and *Rochalimaea* infections and warrants further study in attempts to develop a subunit vaccine to prevent long-term infection by *Rochalimaea* in cats, and the potential for further spread of the organism to humans.

25 A. Cultivation of *Rochalimaea*

Rochalimaea strains were grown on heart infusion agar supplemented with 5% defibrinated rabbit blood (BBL, Cockeysville, Md). The Houston-1 strain of *R. henselae* and the Fuller strain of *R. quintana* were used for all experiments described in this example. Cultures were incubated at 34°C in the presence of 5% CO₂. After growth was sufficient (3 to 4 days) the bacterial cells were harvested with sterile applicators and suspended in TE buffer (10mM Tris pH 8.0, 1mM EDTA). Bacteria were concentrated as

needed by centrifugation. Bacterial cells were then frozen and stored for subsequent immunoblot analysis or the DNA extracted as outlined below.

5 B. Construction of *R. henselae* DNA Library

A lambda phage library of *R. henselae* DNA was prepared using the lambda ZAPII vector (Stratagene, Torrey Pines, Calif.) using a novel method developed specifically for
10 constructing DNA libraries of A-T rich organisms (5). Total (chromosomal and extrachromosomal) DNA was isolated from the Houston-1 strain of *R. henselae* by lysis of the bacterial cells with 1.0% SDS in the presence of proteinase K (100 ng/ μ l) at 55°C for 90 minutes. The resulting lysate was
15 extracted with a 50:50 mixture of phenol and chloroform 4 times. The nucleic acids were precipitated from the aqueous phase by the addition of sodium acetate to 0.3M and 2 1/2 volumes of absolute ethanol. The precipitate was collected by centrifugation and resuspended in TE buffer with 10ng/ μ l
20 of ribonuclease A. The extracted DNA was digested with the restriction endonuclease *Eco*RI under conditions that promote enzymatic activity (star activity). This star activity is less specific and hence more random than the cleavage with *Eco*RI under normal conditions. *Eco*RI star activity generated
25 DNA fragments are efficiently cloned into *Eco*RI-cleaved vectors (5). The resulting DNA was size selected for fragments 3-8 kilobase pair in length by electrophoresis through a 0.7% agarose gel and purification by adsorption to glass milk (GeneClean, Bio101, Vista, Calif.). The purified
30 and sized DNA ligated to *Eco*RI digested alkaline phosphatase-treated lambda ZAPII vector. The recombinant phage concatamers were then packaged into lambda particles using a Gigapack packaging extract (Stratagene, Torrey Pines, Calif.). After packaging, an aliquot of the library was

titered and assayed for the percentage of recombinants by plating with *Escherichia coli* strain XL1 (Stratagene, Torrey Pines, Calif.) on NZY agar plates. The library was amplified by plating the entire packaged ligation reaction and
5 collected by overnight diffusion of the recombinants into phage dilution buffer (10mM Tris pH 8.0, 10mM MgCl₂, 0.1% gelatin).

C. Immunoscreening of recombinant clones

10

For screening of recombinant phage for immunoreactivity with human sera, lambda phage clones were plated at a density of approximately 30,000 per 150 mm plate of NZY agar. After incubation for 3-4 hours at 42°C (until the plaques were
15 barely visible) the plates were overlaid with nitrocellulose filters impregnated with 1 mM isopropyl β -D-thiogalactopyranoside (IPTG) and the incubation continued for an additional 3 hours at 37°C. The filters were then washed twice in Tris-buffered saline containing 0.1% Tween 20, pH
20 8.0 (TBST), and blocked with TBST containing 5.0% dehydrated skim milk (Difco, Detroit, Mich.) for one hour at room temperature. The filters were then reacted with a pool of serum samples (diluted 1:300 in TBST with 5% skim milk) from patients clinically diagnosed with CSD for two hours. This
25 pool of serum samples consisted of 10 individual sera from patients diagnosed with CSD and that were shown to have a titer of 1:1024 or greater as determined by the indirect fluorescent antibody (IFA) assay for *Rochalimaea* performed as previously described (42). The filters were then washed four
30 times with TBST and bound antibody detected by reacting the filters with goat anti-human IgG conjugated with horseradish peroxidase (diluted 1:3000 in TBST with 5% skim milk) for one hour at room temperature. The filters were washed four times in TBST and the color developed using TMB membrane substrate
35 (Kirkegaard and Perry, Gaithersburg, Md).

D. Rescue and analysis of recombinant phagemids

After several rounds of plaque purification of immunoreactive recombinant phage, the isolated phage plaques
5 were subcloned from the lambda phage recombinants into pBluescript-derived plasmids according to the directions of the manufacturer (Stratagene, Torrey Pines, Calif.). Briefly, by superinfecting lambda recombinant-infected *E. coli* XL1 MRF' cells with R408 helper phage, the pBluescript
10 portion of the lambda ZAPII vector containing the inserted *Rochalimaea* DNA was rescued. These recombinant phagemids were then used to infect XL1 MRF' cells, where they replicate as plasmids upon selection with ampicillin. This procedure results in colonies harboring pBluescript-derived recombinant
15 plasmids with *Rochalimaea* DNA inserts. These recombinant plasmids were used for further analysis and sequencing of DNA and for immunoblot analysis of plasmid encoded *Rochalimaea* proteins expressed in *E. coli*.

20

E. Immunoblot analysis

To analyze human antibody response to individual
25 antigens, whole cell lysates of *Rochalimaea* and *E. coli* recombinants were subjected to SDS-PAGE and immunoblot analysis. *Rochalimaea* was grown as described above. *E. coli* recombinants were grown in LB broth containing 100ug/ml of ampicillin at 37°C with shaking to mid-log phase and 1mM IPTG
30 was added. Incubation was continued for an additional 2 hours at 37°C with shaking and cells were collected by centrifugation. The resulting cell pellets were solubilized in sample buffer (2% SDS, 50mM Tris pH 8.0) for SDS-PAGE for 5 minutes at 100°C.

Solubilized *Rochalimaea* and *E. coli* recombinant cell proteins were electrophoresed through 8-16% gradient SDS-PAGE mini gels (Novex, San Diego, Calif.). The resulting cell proteins were stained directly with Coomassie brilliant blue or transferred to nitrocellulose using a mini-transfer apparatus (Bio-Rad, Richmond, Calif.). After electro-transfer of proteins to nitrocellulose, the resulting filters were reacted with antisera and treated as described in the "immunoscreening of recombinant phage clones" section of this Example.

F. Sequencing of phagemid DNA

Plasmid DNA from recombinants was prepared from mini-preps by alkaline lysis. The resulting plasmid DNA was used for restriction endonuclease digestion and directly for double-stranded plasmid sequencing by the dideoxy chain termination method. Plasmids were sequenced using alkaline denaturation by the method of Zhang (44) followed by chain extension and termination with T7 DNA polymerase (Sequenase, US Biochemical) using ³⁵S dATP. Sequenced DNA was electrophoresed through a denaturing 6% acrylamide-urea gel and the resulting gel was fixed with 10% acetic acid and 5% methanol. After vacuum drying, the gel was exposed to X-ray film for approximately 12-24 hours and developed. Plasmids were sequenced with the M13 forward or reverse primers or using primers that were synthesized from the *R. henselae* insert as the sequencing progressed. To further localize the 17-kDa antigen gene and for additional sequencing, restriction fragments were subcloned into pUC19 by standard methods (20) and examined for expression by immunoblot analysis.

G. Construction of fusion protein

Gene fusion techniques were used to confirm the identity of the putative 17-kDa antigen gene and allow for it's optimal expression in *E. coli*. PCR primers from the putative 17-kDa antigen gene sequences were designed that contained a *Hind*III site (underlined) on the 5' end primer (5' AAAAGCTTGAAAAAATATAGCTTAGTCAC 3', SEQ ID NO:13) and a *Bam*HI site (underlined) on the 3' end primer (5' AAGGATCCAGAAATGCTCTCAAAC 3', SEQ ID NO:14). These unique restriction sites facilitate directional and in-frame cloning. DNA from the Houston-1 strain of *R. henselae* was amplified through 35 cycles of 94°C for 1 minutes, 48°C for 2 minutes, and 68°C for 1.5 minutes using GeneAmp reagents and *Taq* polymerase and a thermal cycler (Perkin Elmer, Norwalk, Conn.). The resulting PCR product was cleaved with *Hind*III and *Bam*HI, gel-purified and ligated to the expression vector PinPoint Xa-2 to produce a fusion of the antigen with the 13-kDa biotinylation tag sequence of the vector (Promega, Madison, Wisc.). This tag sequence has been engineered by the manufacturer to permit rapid purification of biotinylated fusion proteins away from other *E. coli* cell proteins. There are also endoprotease cleavage sites to permit cleavage of proteins from the tag sequence. The ligation mixture was used to transform *E. coli* strain XL-1 MRF' (Stratagene, Torrey Pines, Calif.). Potential clones were examined by restriction endonuclease analysis to confirm the presence of the correct size insert. Clones containing the correct size insert were grown to early log-phase in LB broth and induced with 1mM IPTG and growth was allowed to continue an additional two hours. The *E. coli* cells were collected by centrifugation and examined for the expression of biotinylated fusion proteins by immunoblot analysis using streptavidin conjugated to horseradish peroxidase. Clones

expressing the biotinylated fusion protein were examined for reactivity with the serum pool from patients with CSD as well as individual serum samples from patients with CSD and controls. These sera had previously been subjected to IFA to
5 determine the endpoint titer.

H. Identification and Characterization of Immunoreactive Clones

10 Approximately 1.2×10^6 recombinant phage clones were obtained. The quality of the resulting library was previously examined by assaying for cloning efficiency of a marker gene (3). For the purpose of that report, the 16S rRNA gene was chosen and was shown to hybridize with 0.125%
15 of the clones. These results approach those predicted for random cloning and suggests that all DNA fragments should be represented in the *R. henselae* library. Approximately 0.2-0.3% of the 300,000 *R. henselae* recombinant plaques that were screened with the pool of sera from patients diagnosed with
20 CSD, were immunoreactive. Ten of these immunoreactive recombinants were selected for further study and isolated by three or four rounds of plaque-purification. All ten were subcloned as pBluescript-based phagemids and were used to infect *E. coli* strain SOLR (Stratagene, Torrey Pines,
25 Calif.). Each of the ten subclones appear to direct the synthesis of the same 17-kDa antigen that is highly reactive on immunoblots with the pool of sera from patients with CSD. A control strain of *E. coli* XL-1 harboring only the plasmid vector pBluescript SK, did not express the same
30 immunoreactive 17-kDa antigen. *R. henselae* organisms when grown on blood agar appeared to express the 17-kDa antigen weakly, although expression of this antigen by *R. henselae* co-cultivated on Vero cells was not discernable. *R. quintana* also expressed the 17-kDa antigen that reacted with the

pooled sera from CSD patients, suggesting that this antigen (or portions thereof) may be shared between *R. henselae* and *R. quintana*. The 17-kDa antigen expressed by *R. henselae* and the *E. coli* clones did not react with a control human serum pool from patients with no history of CSD. The antibody response from the serum pool did not appear to be directed to variety of different *R. henselae* antigens. Major immunoreactive antigens with approximate molecular masses of 14-, 68-, and 84-kDa are seen with *R. henselae* in addition to minor bands seen at 17-, 32-, and 48-kDa. The 17-kDa antigen expressed in *E. coli* appears to react with the serum pool much stronger than the protein as expressed in *R. henselae*. These results imply that the 17-kDa antigen is highly immunoreactive but not expressed at high levels in *R. henselae*.

I. Analysis of the Gene Encoding the 17-kDa Antigen

To determine if cloning bias was an explanation as to why clones expressing the 17-kDa antigen were isolated at such frequency, restriction endonuclease analysis was performed. Digestion of the plasmids from all ten recombinants expressing the 17-kDa antigen with *EcoRI* revealed seven different profiles. Thus, even though 17-kDa antigen-expressing clones represent the first ten clones isolated, at least seven different DNA fragments contain the entire gene. These results indicate that cloning bias is not responsible for isolating the same antigen-expressing clone by over-representation of a particular fragment of DNA in the library. Accordingly, these results taken together with the results showing few immunoreactive bands for *R. henselae* suggest that the 17-kDa antigen might be one of a few immunodominant antigens recognized by patients with CSD.

The gene for the 17-kDa antigen reactive with the pool of sera from patients with CSD has been localized within a 2.2 kilobase *EcoRI* fragment and the entire fragment has been sequenced. The gene coding for the 17-kDa antigen was

5 localized to a single open reading frame by subcloning and immunoblot analysis with the serum pool from patients with CSD. This gene only directed synthesis of the 17-kDa antigen in *E. coli* when subcloned into pUC19 in the direction of the *lacZ* alpha peptide. Thus, a *R. henselae* promoter is either

10 absent upstream of this gene or non-functional in *E. coli*. The open reading frame either consisted of 160 or 148 amino acids depending on which of the two putative initiator methionines that were identified are functional. The first potential initiator methionine is preceded by a polypurine

15 rich sequence which is similar to the consensus ribosome binding site in *E. coli* (40). The second potential initiator methionine is preceded eight bases by a sequence identical to the consensus *E. coli* ribosome binding site. Assuming the second potential initiator methionine is used for the start

20 of translation, then a deduced protein with 148 amino acids and a predicted molecular weight of 16,893 daltons is obtained. This is closer to the apparent molecular size of 17-kDa that is observed on immunoblots than that which would be predicted (18,245 daltons) if the first potential

25 initiator methionine was used. The deduced amino acid sequence for the 17-kDa antigen gene does not share significant homology with any other bacterial protein in the GenBank database. Assuming the second potential initiator methionine is used, the deduced amino acid sequence shares

30 several similarities to bacterial membrane-associated proteins. The first 18 amino acids are hydrophobic in nature and contain two lysine residues at the immediate amino-terminus (residues two and three). This type of motif is typical of bacterial outer surface proteins where the lysines

residues interact with the phospholipids of the membrane and the following hydrophobic residues form a membrane-spanning or membrane anchor sequence (43). An amino acid sequence identical to the *E. coli* consensus signal peptidase cleavage site (A-X-A) is present at residues 18-20 after the second putative initiator codon. It is not known if this site is cleaved by *R. henselae* signal peptidases to yield a mature form of the 17-kDa antigen. The remaining 130 amino acid residues deduced from the 17-kDa antigen gene sequence are predominantly hydrophilic or neutral, with no long hydrophobic domains.

Regardless of which of the two potential initiator methionines are used for the start of translation, there is a short overlap with another long open reading frame. The reading frame shown upstream of the 17-kDa antigen gene continues at least an additional 380 nucleotides upstream of what is shown. The entire sequence of this open reading frame has not yet been obtained. It is likely that the 17-kDa antigen gene is part of an operon that is transcribed from a promoter upstream of this unidentified open reading frame. Likewise, a second unidentified open reading frame overlaps the 3' end of the 17-kDa antigen gene and continues at least for an additional 700 nucleotides. No sequences that are capable of forming stable loop and stem type secondary structures that are characteristic of bacterial transcription terminators are seen immediately downstream of the 17-kDa antigen gene. This third open reading frame may be a gene that is co-transcribed from a single promoter along with the 17-kDa antigen gene and the potential gene upstream of it. There are no regions upstream of the 17-kDa antigen gene with the correct spacing and homology to the *E. coli* consensus -35 (TTGACA) and -10 (TATAAT) promoter sequences (41). Although, *Rochalimaea* promoter sequences may differ from those of *E. coli*. The lack of potential promoter

sequences and the expression of the 17-kDa antigen only when cloned in the direction of the vector *lacZ* promoter, provides further data that this gene is co-transcribed with an upstream gene.

5

J. Gene Fusion Construction

To create a highly-expressed hybrid fusion protein, the portion of the gene coding for amino acid residues 2-148 were
10 synthesized by PCR amplification using the primers set forth in the Sequence Listing as SEQ ID NO:12 and SEQ ID NO:13. PCR primers were selected that permit in-frame fusion of the entire protein (assuming the second methionine is the start of translation) minus the methionine residue of this gene to
15 the 13-kDa biotinylation tag sequence of expression vector PinPoint Xa-2. This results in a fusion protein which consists of the tag sequence (amino terminal portion) fused to the antigen (carboxy terminal portion) under the control of the *lacZ* promoter of vector pXa-2. The 30-kDa fusion
20 protein is expressed efficiently in *E. coli* as determined by immunoblot analysis with streptavidin conjugated to horseradish peroxidase. A control consisting of an 8-kDa portion of an unrelated gene fused to the pXa-2 vector in *E. coli* strain XL-1 MRF' expresses a biotinylated protein of
25 approximately 21-kDa. A naturally occurring biotinylated protein that has an apparent molecular mass of approximately 26-kDa is found in all three clones. This naturally occurring biotinylated protein has been observed in all K-12 strains of *E. coli* used for recombinant DNA purposes by the
30 vector manufacturer (Promega Corp., Madison, Wisc.).

K. Immunoreactivity of the fusion protein

To examine the reactivity of the 30-kDa fusion protein with individual serum samples from patients with CSD as well
5 as controls, immunoblot analysis was performed. When individual serum samples were reacted with the proteins expressed by the clone containing the fusion protein, 12 of 13 serum samples that were positive for antibody to *Rochalimaea* by the IFA assay were also reactive with the 30-
10 kDa recombinant fusion protein. Likewise, 8 of 9 serum samples that were negative by IFA did not react by immunoblot with the 30-kDa fusion protein. When the immunoblot strips are examined only for reactivity of the 30-kDa fusion protein there is good correlation with clinical diagnosis of CSD and
15 IFA titer. These results also indicate that a dominant epitope of the 17-kDa antigen recognized by serum from patients with CSD is retained in the fusion protein.

Throughout this application various publications are
20 referenced by numbers within parentheses. Full citations for these publications are as follows. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention
25 pertains.

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80

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Anderson, Burt E.
Regnery, Russell L
- (ii) TITLE OF INVENTION: Nucleic Acids of Rochalimaea Henselae
and Methods and Compositions for Diagnosing Rochalimaea
Henselae and Rochalimaea Quintana Infection
- (iii) NUMBER OF SEQUENCES: 14
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: NEEDLE & ROSENBERG, P.C.
 - (B) STREET: 127 Peachtree Street, N.E., Suite 1200
 - (C) CITY: Atlanta
 - (D) STATE: Georgia
 - (E) COUNTRY: USA
 - (F) ZIP: 30303
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Spratt, Gwendolyn D.
 - (B) REGISTRATION NUMBER: 36,016
 - (C) REFERENCE/DOCKET NUMBER: 1414.624
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (404) 688-0770
 - (B) TELEFAX: (404) 688-9880

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GATTCAATTG GTTTGAAGGA GGCT

24

(2) INFORMATION FOR SEQ ID NO:2:

81

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATTCAATTG GTTTGAAAGA GGCT

24

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCACATCACC AGGACGTATT C

21

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCACATCACC AGGGCGTATT C

21

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTGC GTTAA TTACCGATCC

20

(2) INFORMATION FOR SEQ ID NO:6:

82

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGCGCTTTGA TTACTGATCC

20

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1791 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 141..1652

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAAGAGCAAT AAAAAGAAAA AAGAATGGTT TTTTAGTGAT TTTTTTAGTA CTCCAATTTA	60
GACAGAAAAC GGTAAGGTTT GTTATTTTAT AAAGGACTGC AATTGGGATA ACAATATGAT	120
TAAATAGGAG CACATACCAA ATG GTT AAA AAA ACT TTC TTC ACA ACA TTA	170
Met Val Lys Lys Thr Phe Phe Thr Thr Leu	
1 5 10	
GCC GCA GTA AGT TTT TCT GCT GCT TTA GAA ACT GCA CTG TTT TTT AGT	218
Ala Ala Val Ser Phe Ser Ala Ala Leu Glu Thr Ala Leu Phe Phe Ser	
15 20 25	
GGA TGT GGA TCA AGC TTG TGG ACG ACA AAA GCT CAT GCA AAT TCT GTA	266
Gly Cys Gly Ser Ser Leu Trp Thr Thr Lys Ala His Ala Asn Ser Val	
30 35 40	
TTT AGT TCA TTA ATG CAA CAG CAG GGA TTT GCA GAT ATT GTT TCT CAA	314
Phe Ser Ser Leu Met Gln Gln Gly Phe Ala Asp Ile Val Ser Gln	
45 50 55	
GTA AAG CCT GCT GTT GTT TCA GTG CAG GTG AAG AGC AAT AAA AAG AAA	362
Val Lys Pro Ala Val Val Ser Val Gln Val Lys Ser Asn Lys Lys Lys	
60 65 70	
AAA GAA TGG TTT TTT AGT GAT TTT TTT AGT ACT CCG GGT TTT GAC CAA	410
Lys Glu Trp Phe Phe Ser Asp Phe Phe Ser Thr Pro Gly Phe Asp Gln	
75 80 85 90	
TTA CCA GAT CAA CAT CCC TTG AAA AAG TTT TTT CAA GAT TTT TAT AAT	458
Leu Pro Asp Gln His Pro Leu Lys Lys Phe Phe Gln Asp Phe Tyr Asn	
95 100 105	

83

CGT GAT AAG CCT AGT AAT AAA TCT TTG CAA CGT TCG CAT AGA CTG CGT Arg Asp Lys Pro Ser Asn Lys Ser Leu Gln Arg Ser His Arg Leu Arg 110 115 120	506
CCT ATA GCT TTT GGA TCG GGT TTT TTT ATC TCG TCT GAT GGT TAT ATT Pro Ile Ala Phe Gly Ser Gly Phe Phe Ile Ser Ser Asp Gly Tyr Ile 125 130 135	554
GTG ACC AAT AAT CAT GTG ATT TCT GAT GGC ACA AGT TAC GCT GTT GTT Val Thr Asn Asn His Val Ile Ser Asp Gly Thr Ser Tyr Ala Val Val 140 145 150	602
CTT GAT GAC GGT ACA GAA CTG AAT GCA AAA CTC ATT GGA ACG GAC CCA Leu Asp Asp Gly Thr Glu Leu Asn Ala Lys Leu Ile Gly Thr Asp Pro 155 160 165 170	650
CGA ACT GAT CTT GCA GTA TTA AAA GTC AAT GAA AAA AGA AAA TTT TCG Arg Thr Asp Leu Ala Val Leu Lys Val Asn Glu Lys Arg Lys Phe Ser 175 180 185	698
TAC GTT GAT TTT GGT GAT GAT TCA AAA CTT CGT GTT GGT GAT TGG GTT Tyr Val Asp Phe Gly Asp Asp Ser Lys Leu Arg Val Gly Asp Trp Val 190 195 200	746
GTT GCT ATT GGT AAT CCA TTT GGT CTT GGT GGA ACT GTG ACA GCA GGT Val Ala Ile Gly Asn Pro Phe Gly Leu Gly Gly Thr Val Thr Ala Gly 205 210 215	794
ATC GTT TCA GCA CGT GGA CGT GAT ATC GGT ACC GGT GTT TAT GAT GAT Ile Val Ser Ala Arg Gly Arg Asp Ile Gly Thr Gly Val Tyr Asp Asp 220 225 230	842
TTT ATT CAG ATT GAT GCT GCA GTT AAT CGA GGA AAT TCT GGA GGT CCA Phe Ile Gln Ile Asp Ala Ala Val Asn Arg Gly Asn Ser Gly Gly Pro 235 240 245 250	890
ACT TTT GAT CTT AAC GGA AAG GTT GTT GGA GTG AAT ACG GCA ATT TTT Thr Phe Asp Leu Asn Gly Lys Val Val Gly Val Asn Thr Ala Ile Phe 255 260 265	938
TCT CCT TCT GGG GGC AAC GTT GGG ATT GCT TTC GCT ATT CCG GCA GCA Ser Pro Ser Gly Gly Asn Val Gly Ile Ala Phe Ala Ile Pro Ala Ala 270 275 280	986
ACA GCG AAC GAG GTT GTG CAA CAA CTT ATC GAA AAA GGT TTA GTT CAG Thr Ala Asn Glu Val Val Gln Gln Leu Ile Glu Lys Gly Leu Val Gln 285 290 295	1034
CGT GGT TGG CTT GGG GTT CAG ATT CAG CCT GTA ACA AAA GAA ATT TCT Arg Gly Trp Leu Gly Val Gln Ile Gln Pro Val Thr Lys Glu Ile Ser 300 305 310	1082
GAT TCA ATT GGT TTG AAG GAG GCT AAA GGT GCG TTA ATT ACC GAT CCA Asp Ser Ile Gly Leu Lys Glu Ala Lys Gly Ala Leu Ile Thr Asp Pro 315 320 325 330	1130
TTA AAG GGG CCA GCC GCA AAA GCT GGT ATC AAG GCA GGT GAT GTT ATT Leu Lys Gly Pro Ala Ala Lys Ala Gly Ile Lys Ala Gly Asp Val Ile 335 340 345	1178
ATT TCG GTA AAT GGT GAG AAG ATT AAT GAT GTC CGT GAT CTA GCA AAG Ile Ser Val Asn Gly Glu Lys Ile Asn Asp Val Arg Asp Leu Ala Lys 350 355 360	1226

84

CGT ATT GCA AAT ATG AGC CCA GGA GAA ACA GTA ACC TTA GGA GTT TGG Arg Ile Ala Asn Met Ser Pro Gly Glu Thr Val Thr Leu Gly Val Trp 365 370 375	1274
AAA TCT GGT AAA GAA GAG AAT ATT AAG GTT AAA CTT GAT TCG ATG CCT Lys Ser Gly Lys Glu Glu Asn Ile Lys Val Lys Leu Asp Ser Met Pro 380 385 390	1322
GAA GAC GAA AAT ATG AAG GAT GGC TCA AAA TAT TCA AAT GAG CAC GGT Glu Asp Glu Asn Met Lys Asp Gly Ser Lys Tyr Ser Asn Glu His Gly 395 400 405 410	1370
AAT TCA GAT GAA ACA TTG GAA GAT TAT GGT TTG ATT GTT GCT CCT TCT Asn Ser Asp Glu Thr Leu Glu Asp Tyr Gly Leu Ile Val Ala Pro Ser 415 420 425	1418
GAT GAT GGC CTA GGG TTG GTT GTA ACT GAT GTA GAT CCA GAT TCT GAT Asp Asp Gly Leu Gly Leu Val Val Thr Asp Val Asp Pro Asp Ser Asp 430 435 440	1466
GCT GCA GAT AAA GGA ATA CGT CCT GGT GAT GTG ATT GTA ACA GTT AAT Ala Ala Asp Lys Gly Ile Arg Pro Gly Asp Val Ile Val Thr Val Asn 445 450 455	1514
AAT AAA TCT GTT AAA AAG GTC TCT GAT ATT ACG GAC ACT ATC AAA AAT Asn Lys Ser Val Lys Lys Val Ser Asp Ile Thr Asp Thr Ile Lys Asn 460 465 470	1562
GCC CAA AAG TTA GGA CGA AAA GCC ATA CTT CTA CAA GTG CGA ACA AAT Ala Gln Lys Leu Gly Arg Lys Ala Ile Leu Leu Gln Val Arg Thr Asn 475 480 485 490	1610
GAT CAA AAT CGT TTT GTC GCT CTT CCT ATT TTT AAA AAA TAATACTTGA Asp Gln Asn Arg Phe Val Ala Leu Pro Ile Phe Lys Lys 495 500	1659
TTAATGGTAG GGCAGAAAGTT TTGTAACTT TTGTCCTACA AACGTGATTT GATAAAATAA	1719
CGGAGATGCG TTTTATGAAG ATACTCGTTA TCGAAGATGA TCATGAAACG GGACGTTATC	1779
TCGAAAAGCT TT	1791

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 503 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Val Lys Lys Thr Phe Phe Thr Thr Leu Ala Ala Val Ser Phe Ser 1 5 10 15
Ala Ala Leu Glu Thr Ala Leu Phe Phe Ser Gly Cys Gly Ser Ser Leu 20 25 30
Trp Thr Thr Lys Ala His Ala Asn Ser Val Phe Ser Ser Leu Met Gln 35 40 45

85

Gln Gln Gly Phe Ala Asp Ile Val Ser Gln Val Lys Pro Ala Val Val
 50 55 60
 Ser Val Gln Val Lys Ser Asn Lys Lys Lys Lys Glu Trp Phe Phe Ser
 65 70 75 80
 Asp Phe Phe Ser Thr Pro Gly Phe Asp Gln Leu Pro Asp Gln His Pro
 85 90 95
 Leu Lys Lys Phe Phe Gln Asp Phe Tyr Asn Arg Asp Lys Pro Ser Asn
 100 105 110
 Lys Ser Leu Gln Arg Ser His Arg Leu Arg Pro Ile Ala Phe Gly Ser
 115 120 125
 Gly Phe Phe Ile Ser Ser Asp Gly Tyr Ile Val Thr Asn Asn His Val
 130 135 140
 Ile Ser Asp Gly Thr Ser Tyr Ala Val Val Leu Asp Asp Gly Thr Glu
 145 150 155 160
 Leu Asn Ala Lys Leu Ile Gly Thr Asp Pro Arg Thr Asp Leu Ala Val
 165 170 175
 Leu Lys Val Asn Glu Lys Arg Lys Phe Ser Tyr Val Asp Phe Gly Asp
 180 185 190
 Asp Ser Lys Leu Arg Val Gly Asp Trp Val Val Ala Ile Gly Asn Pro
 195 200 205
 Phe Gly Leu Gly Gly Thr Val Thr Ala Gly Ile Val Ser Ala Arg Gly
 210 215 220
 Arg Asp Ile Gly Thr Gly Val Tyr Asp Asp Phe Ile Gln Ile Asp Ala
 225 230 235 240
 Ala Val Asn Arg Gly Asn Ser Gly Gly Pro Thr Phe Asp Leu Asn Gly
 245 250 255
 Lys Val Val Gly Val Asn Thr Ala Ile Phe Ser Pro Ser Gly Gly Asn
 260 265 270
 Val Gly Ile Ala Phe Ala Ile Pro Ala Ala Thr Ala Asn Glu Val Val
 275 280 285
 Gln Gln Leu Ile Glu Lys Gly Leu Val Gln Arg Gly Trp Leu Gly Val
 290 295 300
 Gln Ile Gln Pro Val Thr Lys Glu Ile Ser Asp Ser Ile Gly Leu Lys
 305 310 315 320
 Glu Ala Lys Gly Ala Leu Ile Thr Asp Pro Leu Lys Gly Pro Ala Ala
 325 330 335
 Lys Ala Gly Ile Lys Ala Gly Asp Val Ile Ile Ser Val Asn Gly Glu
 340 345 350
 Lys Ile Asn Asp Val Arg Asp Leu Ala Lys Arg Ile Ala Asn Met Ser
 355 360 365
 Pro Gly Glu Thr Val Thr Leu Gly Val Trp Lys Ser Gly Lys Glu Glu
 370 375 380

86

Asn Ile Lys Val Lys Leu Asp Ser Met Pro Glu Asp Glu Asn Met Lys
 385 390 395 400
 Asp Gly Ser Lys Tyr Ser Asn Glu His Gly Asn Ser Asp Glu Thr Leu
 405 410 415
 Glu Asp Tyr Gly Leu Ile Val Ala Pro Ser Asp Asp Gly Leu Gly Leu
 420 425 430
 Val Val Thr Asp Val Asp Pro Asp Ser Asp Ala Ala Asp Lys Gly Ile
 435 440 445
 Arg Pro Gly Asp Val Ile Val Thr Val Asn Asn Lys Ser Val Lys Lys
 450 455 460
 Val Ser Asp Ile Thr Asp Thr Ile Lys Asn Ala Gln Lys Leu Gly Arg
 465 470 475 480
 Lys Ala Ile Leu Leu Gln Val Arg Thr Asn Asp Gln Asn Arg Phe Val
 485 490 495
 Ala Leu Pro Ile Phe Lys Lys
 500

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AATCTAGATT GCTTTCGCTA TTCCGGC

27

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGGATCCAT TTGTTCGCAC TTGTAGAAG

29

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 660 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double

87

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 123..605

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

TCCGTTGTTG CAGAACTTAA CTTACGTGGA ATGAATGATG AGATCGCCGT CTTAAGCGGT      60
ACAACAAAGA ATATTGAACT GGTGAACCAA ATTATCAGCG AATATGGAGC AGACCCAGAC      120
AT ATG GCT GCC TAT ATT TCA TCA AAG GAG AGA AAA TCA ATG AAA AAA      167
Met Ala Ala Tyr Ile Ser Ser Lys Glu Arg Lys Ser Met Lys Lys
  1             5             10             15

TAT AGC TTA GTC ACA TTG TTA TCT TTA TTT TGC ATC TCT CAT GCA AAA      215
Tyr Ser Leu Val Thr Leu Leu Ser Leu Phe Cys Ile Ser His Ala Lys
             20             25             30

GCA CAA ACA GCA ACC CTT ACT GAT GAA TAT TAT AAA AAA GCC TTA GAA      263
Ala Gln Thr Ala Thr Leu Thr Asp Glu Tyr Tyr Lys Lys Ala Leu Glu
             35             40             45

AAC ACG CAA AAA TTA GAC GTT GCA AAA TCA CAA ACA GCT GAG TCT ATT      311
Asn Thr Gln Lys Leu Asp Val Ala Lys Ser Gln Thr Ala Glu Ser Ile
             50             55             60

TAT GAA TCT GCA ACA CAA ACT GCA AAC AAA ATT AAG GAC ATA AAC AAT      359
Tyr Glu Ser Ala Thr Gln Thr Ala Asn Lys Ile Lys Asp Ile Asn Asn
             65             70             75

CAA CTT GCA AAT CTT AAA GCA GAT ACA AAG ACT AAA CCT GAA CAA TTG      407
Gln Leu Ala Asn Leu Lys Ala Asp Thr Lys Thr Lys Pro Glu Gln Leu
             80             85             90             95

CAA GCC CTG CAA ATA GAG CTG ACT CTT CTC CAG GCA CAG CTG CAA GCG      455
Gln Ala Leu Gln Ile Glu Leu Thr Leu Leu Gln Ala Gln Leu Gln Ala
             100             105             110

GAT ACT TTA AAA ATC CAG TCT CTT GCT ATG ATT CAA GCA AAA GAT ACG      503
Asp Thr Leu Lys Ile Gln Ser Leu Ala Met Ile Gln Ala Lys Asp Thr
             115             120             125

AAA ACA AAA GAA GAA TTG CGT GAA GAG CAA ACA CAA AAA AAG CAT GAA      551
Lys Thr Lys Glu Glu Leu Arg Glu Glu Gln Thr Gln Lys Lys His Glu
             130             135             140

GAT CTT CAA AAA CAA TTA AAA GAA AAA CTT GAG AAA TCT GAT GTC CGA      599
Asp Leu Gln Lys Gln Leu Lys Glu Lys Leu Glu Lys Ser Asp Val Arg
             145             150             155

CTT TAGTTTTTCC CCGTTTGAGA GCATTTCTGG ATATATTTTA CAACCACTCA ATAATGTA
660
Leu
160

```

88

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 160 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

Met Ala Ala Tyr Ile Ser Ser Lys Glu Arg Lys Ser Met Lys Lys Tyr
 1           5           10           15
Ser Leu Val Thr Leu Leu Ser Leu Phe Cys Ile Ser His Ala Lys Ala
          20           25           30
Gln Thr Ala Thr Leu Thr Asp Glu Tyr Tyr Lys Lys Ala Leu Glu Asn
          35           40           45
Thr Gln Lys Leu Asp Val Ala Lys Ser Gln Thr Ala Glu Ser Ile Tyr
          50           55           60
Glu Ser Ala Thr Gln Thr Ala Asn Lys Ile Lys Asp Ile Asn Asn Gln
          65           70           75           80
Leu Ala Asn Leu Lys Ala Asp Thr Lys Thr Lys Pro Glu Gln Leu Gln
          85           90           95
Ala Leu Gln Ile Glu Leu Thr Leu Leu Gln Ala Gln Leu Gln Ala Asp
          100          105          110
Thr Leu Lys Ile Gln Ser Leu Ala Met Ile Gln Ala Lys Asp Thr Lys
          115          120          125
Thr Lys Glu Glu Leu Arg Glu Glu Gln Thr Gln Lys Lys His Glu Asp
          130          135          140
Leu Gln Lys Gln Leu Lys Glu Lys Leu Glu Lys Ser Asp Val Arg Leu
          145          150          155          160

```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AAAAGCTTGA AAAAATATAG CTTAGTCAC

29

(2) INFORMATION FOR SEQ ID NO:14:

89

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAGGATCCAG AAATGCTCTC AAAC

24

What is claimed is:

1. An isolated nucleic acid encoding the amino acid sequence set forth in the Sequence Listing as SEQ ID NO:11.
2. The isolated nucleic acid of Claim 1 in a vector suitable for expressing the nucleic acid.
3. The vector of Claim 2 in a host suitable for expressing the nucleic acid.
4. An isolated nucleic acid, of at least 15 nucleotides in length, which can selectively hybridize with the nucleic acid of Claim 1, under polymerase chain reaction conditions.
5. An isolated nucleic acid, of at least 15 nucleotides in length, which can specifically hybridize with the nucleic acid of Claim 1, under the stringency conditions of 60°C and 5X SSC, followed by the initial washing condition of room temperature, 2X SSC and 0.1% SDS, and two secondary washes with stringency conditions of 50°C, 0.5% SSC and 0.1% SDS.
6. A purified antigenic polypeptide encoded by the isolated nucleic acid of Claim 5.
7. A purified antigenic polypeptide having the amino acid sequence set forth in the Sequence Listing as SEQ ID NO:11.
8. An isolated nucleic acid consisting of the nucleotides in the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:7.
9. The nucleic acid of Claim 8 in a vector suitable for expressing the nucleic acid.
10. The vector of Claim 9 in a host suitable for expressing the nucleic acid.

11. An isolated nucleic acid that specifically hybridizes with the nucleic acid of Claim 8 under high stringency conditions and at least 85% sequence complementarity with the segment of the strand to which it hybridizes.

12. A purified antigenic protein encoded by the nucleic acid of Claim 8.

13. An antigenic polypeptide fragment encoded by the nucleic acid of Claim 8.

14. An isolated nucleic acid specific for *Rochalimaea henselae*, comprising the nucleotides in the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:5.

15. An isolated nucleic acid specific for *Rochalimaea quintana*, comprising the nucleotides in the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:6.

16. A mixture of the nucleic acids set forth in the sequence listing as SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4.

17. A method of diagnosing current or previous cat scratch disease in a subject, comprising detecting the presence of *Rochalimaea henselae* in a sample from the subject, wherein the detecting step comprises the steps of contacting a fluid or tissue sample from the subject with a detectable amount of a purified antibody that specifically binds the polypeptide set forth in the Sequence Listing as SEQ ID NO:11, detecting the binding of the antibody to polypeptide, and correlating the presence of specific binding with cat scratch disease in the subject.

18. A method of diagnosing current or previous cat scratch disease in a subject, comprising detecting the presence of *Rochalimaea henselae* in a sample from the subject, wherein

the detecting step comprises the steps of contacting a fluid or tissue sample from the subject with a detectable amount of a purified antibody that specifically binds the polypeptide set forth in the Sequence Listing as SEQ ID NO:7, detecting the binding of the antibody to polypeptide, and correlating the presence of specific binding with cat scratch disease in the subject.

19. A method of diagnosing current or previous cat scratch disease in a subject by detecting the presence of an antibody that specifically binds *R. henselae*, wherein the detecting step comprises the steps of contacting an antibody-containing fluid or tissue sample from the subject with an amount of the purified polypeptide set forth in the Sequence Listing as SEQ ID NO:7, detecting the binding of the polypeptide to the antibody, and correlating the presence of an antibody that specifically binds to the polypeptide with cat scratch disease in the subject.

20. A method of diagnosing current or previous cat scratch disease in a subject by detecting the presence of an antibody that specifically binds *R. henselae*, wherein the detecting step comprises the steps of contacting an antibody-containing fluid or tissue sample from the subject with an amount of the purified polypeptide set forth in the Sequence Listing as SEQ ID NO:11, detecting the binding of the polypeptide to the antibody, and correlating the presence of an antibody that specifically binds to the polypeptide with cat scratch disease in the subject.

21. A method of diagnosing current or previous cat scratch disease in a subject by detecting the presence of an antibody that specifically binds *R. henselae*, wherein the detecting step comprises the steps of contacting an antibody-containing fluid or tissue sample from the subject with an amount of the purified polypeptide encoded by the nucleic acid which can specifically hybridize with the nucleic acid encoding the

amino acid sequence set forth in the Sequence Listing as SEQ ID NO:11 under the stringency conditions of 60°C and 5X SSC, followed by the initial washing condition of room temperature, 2X SSC and 0.1% SDS, and two secondary washes with stringency conditions of 50°C, 0.5% SSC and 0.1% SD, detecting the binding of the polypeptide to the antibody, and correlating the presence of an antibody that specifically binds to the polypeptide with cat scratch disease in the subject.

22. A method of diagnosing current or previous cat scratch disease in a subject, comprising detecting the presence of *Rochalimaea henselae* in a sample from the subject, wherein the presence of *Rochalimaea henselae* is determined by detecting the presence of a nucleic acid encoding the amino acid sequence set forth in the Sequence Listing as SEQ ID NO:11.

23. The method of Claim 22, wherein the nucleic acid is detected utilizing a nucleic acid amplification technique.

24. The method of Claim 22, wherein the subject is a human.

25. A method of diagnosing bacillary angiomatosis in a subject comprising detecting the presence of *Rochalimaea henselae* in a sample from the subject, wherein the presence of *Rochalimaea henselae* is determined by detecting the presence of a nucleic acid encoding the amino acid sequence set forth in the Sequence Listing as SEQ ID NO:5, and correlating the presence of *Rochalimaea henselae* with bacillary angiomatosis in the subject.

26. A method of diagnosing bacillary angiomatosis in a subject comprising detecting the presence of *Rochalimaea quintana* in a sample from the subject by detecting the presence of a nucleic acid specific for *Rochalimaea quintana*, wherein the nucleic acid specific for *Rochalimaea quintana*

consists of the nucleotides in the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:6, and correlating the presence of *Rochalimaea quintana* with the presence of bacillary angiomatosis in the subject.

27. A method of diagnosing bacillary angiomatosis in a subject comprising detecting the presence of *Rochalimaea quintana* in a sample from the subject by detecting the presence of a nucleic acid specific for *Rochalimaea quintana*, wherein the nucleic acid specific for *Rochalimaea quintana* consists of the nucleotides in the nucleotide sequence defined in the Sequence Listing as SEQ ID NO:11, and correlating the presence of *Rochalimaea quintana* with the presence of bacillary angiomatosis in the subject.

28. A method of diagnosing cat scratch disease or bacillary angiomatosis in a subject, comprising: amplifying DNA from the subject using a primer mixture consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4; contacting the amplified DNA from step a. with a probe consisting of the nucleic acid of SEQ ID NO:5 and detecting the hybridization of the probe with the amplified DNA, the existence of hybridization indicating the presence of *R. henselae*, which is correlated with cat scratch disease; and contacting the amplified DNA from step a. with a probe consisting of the nucleic acid of SEQ ID NO:6 and detecting the hybridization of the probe with the amplified DNA, the existence of hybridization indicating the presence of *R. quintana*, which is correlated with bacillary angiomatosis.

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ROCHALI- MAEA HENSELAE	(19)	10/34	16/32	8/32
ROCHALI- MAEA VINSONII	10.8	(15)	8/28	6/28
ROCHALI- MAEA QUINTANA	6.0	11.0	(13)	8/26
RICKETT- SIA PRO- WAZEKII	12.2	13.6	10.3	(13)

FIG.1

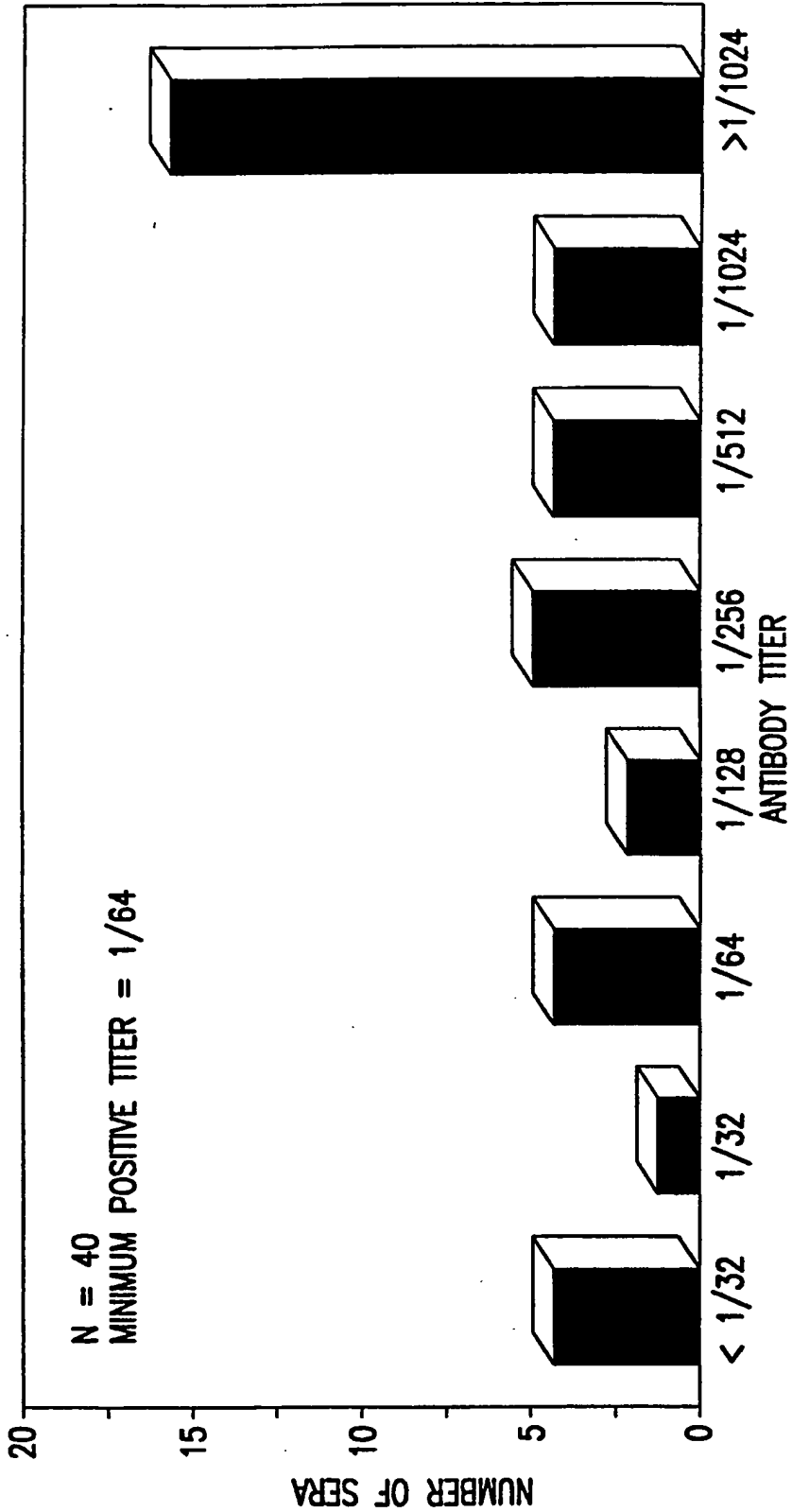


FIG.2

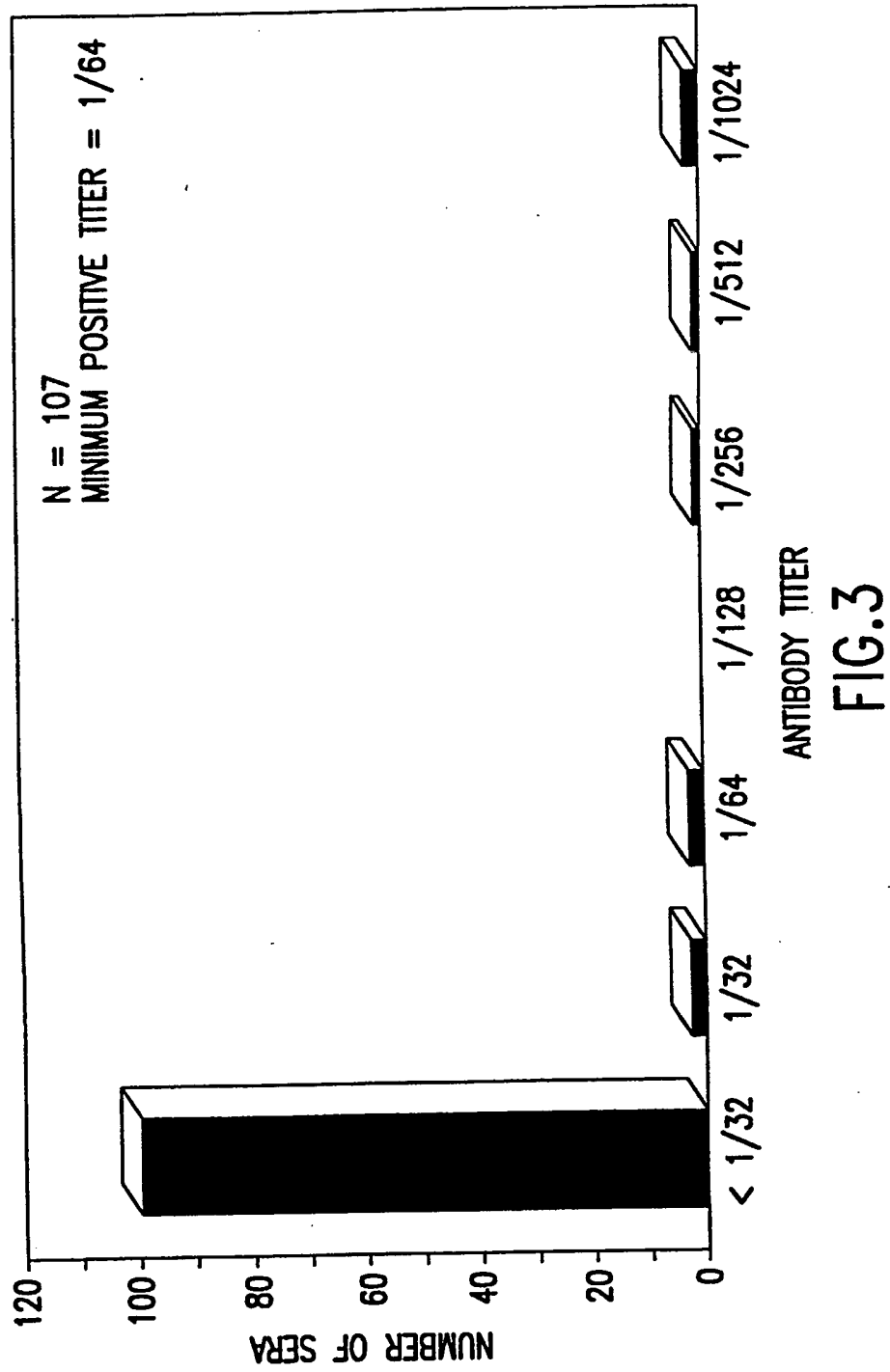


FIGURE 4

Species	CAT1	RH1/RQ1	CAT2 (C)
<i>R. henselae</i>	GATTCAATTGGTTTGAAGGAGGCT (SEQ ID NO:1)	GGTGGTTAATTACCGATCC (SEQ ID NO:5)	GAATACGTCCTGGTGATGTGA (SEQ ID NO:3 complement)
<i>R. quintana</i>A..... (SEQ ID NO:2)	..C..T..G.....T..... (SEQ ID NO:6)C..... (SEQ ID NO:4 complement)
<i>R. elizabethae</i>G..A.....C.A..A..AT.NGG.G..T.....	ND
<i>R. vinsonii</i>	..C.....A.....A....ACT.....C..T.....	ND

INTERNATIONAL SEARCH REPORT

Internat'l Application No

PCT/US 95/06211

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C12N15/70 C07K14/29 G01N33/569 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF CLINICAL MICROBIOLOGY, vol. 32, no. 4, April 1994 pages 942-948, BURT ANDERSON ET AL. 'Detection of Rochalimaea henselae DNA in specimens from cat scratch disease patients by PCR' see page 943, left column, paragraph 2 see page 946, left column, paragraph 2 - right column, paragraph 2	7-11, 14, 25
Y	WO-A-93 14406 (THE UNITED STATES OF AMERICA) 22 July 1993 see page 5, line 3 - page 7, line 2 see page 11, line 14 - line 19 see page 13, line 30 - page 14, line 23 see page 25, line 5 - page 28, line 6 --- -/--	8-14, 18, 19, 25

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/06211

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBL Database entry RHHTRA Accession number L20127; 30 June 1993 ANDERSON B. ET AL.: 'Rochalimaea henselae antigen (htrA) gene' ----	8-14, 18, 19, 25
A	ABSTRACTS OF THE 93RD GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, 16 May 1993 - 20 May 1993 WASHINGTON US, page 111 B. ANDERSON ET AL. 'Molecular cloning of Rochalimaea henselae antigens' cited in the application see abstract no. D-90 -----	8-14, 18, 19, 25

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern: al Application No

PCT/US 95/06211

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9314406	22-07-93	US-A- 5399485	21-03-95
		AU-B- 3484993	03-08-93
		CA-A- 2127470	22-07-93
		EP-A- 0623215	09-11-94
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